Bioenergetics of Methanogenesis from Acetate by Methanosarcina barkeri†

SUSANNE PEINEMANN, VOLKER MÜLLER, MICHAEL BLAUT, AND GERHARD GOTTSCALK*

Institut für Mikrobiologie der Universität Göttingen, Grisebachstrasse 8, D-3400 Göttingen, Federal Republic of Germany

Received 24 June 1987/Accepted 17 November 1987

Methane formation from acetate by resting cells of Methanosarcina barkeri was accompanied by an increase in the intracellular ATP content from 0.9 to 4.0 nmol/mg of protein. Correspondingly, the proton motive force increased to a steady-state level of −120 mV. The transmembrane pH gradient, however, was reversed under these conditions and amounted to +20 mV. The addition of the protonophore 3,5,3′,4′-tetrachlorosilacylanilide led to a drastic decrease in the proton motive force and in the intracellular ATP content and to an inhibition of methane formation. The ATPase inhibitor N,N′-dicyclohexylcarbodiimide stopped methanogenesis, and the intracellular ATP content decreased. The proton motive force decreased also under these conditions, indicating that the proton motive force could not be generated from acetate without ATP. The overall process of methane formation from acetate was dependent on the presence of sodium ions; upon addition of acetate to cell suspensions of M. barkeri, a transmembrane Na⁺ gradient in the range of 4.1 (Na⁺o/Na⁺i) was established. Possible sites of involvement of the Na⁺ gradient in the conversion of acetate to methane and carbon dioxide are discussed. Na⁺ is not involved in the CO dehydrogenase reaction.

Recently, it was shown that methanogenesis from H₂ plus methanol, H₂ plus trimethylamine, and H₂ plus formaldehyde by resting cells of Methanosarcina barkeri is coupled to ATP formation by a chemiosmotic mechanism (2, 3, 19). This conclusion was based on the following findings. (i) The addition of the protonophore tetrachlorosilacylanilide (TCS) to resting cells of M. barkeri forming methane from methanol plus H₂, formaldehyde plus H₂, or trimethylamine plus H₂ caused a dissipation of the proton motive force (Δp) and a decrease in the intracellular ATP content, whereas methane formation was stimulated. (ii) The ATPase inhibitor N,N′-dicyclohexylcarbodiimide (DCCD) inhibited methane formation and ATP synthesis but left Δp intact. (iii) Addition of TCS to cell suspensions incubated with DCCD restored their ability to form methane. We have extended these studies now to cells of M. barkeri grown on acetate. Important differences have been encountered in comparison to the substrate combination methanol plus H₂; they are reported in this publication.

It has been known for some time now that the growth of methanogenic bacteria and methane formation depend on sodium ions (20); an involvement of Na⁺ in the process of ATP synthesis was envisaged. However, we have shown that ATP synthesis during methanogenesis from methanol plus H₂ does not depend on the presence of Na⁺ (4). Recently, one site of Na⁺ involvement was identified. Na⁺ is required for methyl group oxidation during dismutation of methanol to methane and carbon dioxide (4). It was of interest to study the effect of Na⁺ on methanogenesis from acetate.

**MATERIALS AND METHODS**

*M. barkeri* Fusaro (DSM 804) was grown on 100 mM sodium acetate as the sole carbon and energy source. The medium described by Hippe et al. (8) was prepared by the anaerobic techniques of Hungate (9) as described recently (19). The gas atmosphere was N₂-CO₂ (80:20, vol/vol). Growing on sodium acetate, *M. barkeri* had a doubling time of 24 h and the growth yield was 2.5 mg (cell dry weight)/mmol of methane formed.

Cell suspensions of *M. barkeri* in 100 mM sodium pipazine-N,N′-bis(2-ethanesulfonic acid) (sodium PIPES) buffer, pH 6.7, containing 2 ml of titanium(III) citrate solution were prepared as described previously (19). The protein concentration of the final cell suspension was 13 to 14 mg/ml. For experiments in which the sodium dependence was tested, cell suspensions were prepared in 20 mM imidazole buffer, pH 6.8, supplemented with 25 mM KCl. The protein content was determined by the method of Schmidt et al. (22).

**Experimental conditions.** Effects of DCCD and TCS were studied as described previously (19). To study the effect of sodium ions, 20 mM potassium acetate served as the substrate and NaCl was added as indicated with a syringe from a 3 M stock solution. H₂ formation from CO plus H₂O by cell suspensions was measured in the presence of 20 mM potassium 2-bromothanesulfonate (BES)-1 mM propyl iodide-20 mM KCl in the absence of light (5). The reaction was started by the addition of 5 ml of gaseous CO. A potassium BES solution was prepared from the sodium salt by ion-exchange chromatography on Dowex 50 WX8. Fifty milliliters of 0.2 M sodium BES was mixed with 100 ml of Dowex 50 WX8, 20/50 mesh, and incubated with gentle stirring for 15 min. The BES solution was subsequently separated from the resin by filtration. The resulting solution was passed twice through a 5-ml column filled with Dowex 50 WX8, 100/200 mesh. Finally, the pH of the BES solution was adjusted with KOH to 7.0. The sodium concentration in this solution was below 1 mM.

Methane, acetate, and H₂ concentrations were determined by gas chromatography (4, 17), and ATP concentration was determined by the luciferin luciferase assay as described previously (13, 19).

Δψ and ΔpH were estimated from the transmembrane equilibrium distribution of the lipophilic cation [¹⁴C]tetraphenylphosphonium bromide and the weak acid [¹³C]-
benzoic acid, respectively, by the method of Rottenberg (21) as described previously (19).

**Determination of the extracellular and intracellular sodium concentrations.** A portion of the cell suspension was freed of cells by centrifugation. The sodium concentration was measured with a sodium electrode (Orion Research AG, Küssnacht, Switzerland) connected to an ion meter (Orion Research AG). The intracellular Na\(^+\) concentration was determined by using a membrane filtration method. Cells in the mid-log phase were harvested as described above and suspended in 20 mM imidazole hydrochloride buffer, pH 6.5, containing 20 mM MgCl\(_2\) and 25 mM NaCl. Experiments were performed anerobically under N\(_2\) at 37°C in 18-ml serum tubes with 1 ml of the buffer described above. A 100-\(\mu\)l portion of the concentrated cell suspension was added anaerobically. After preincubation for 15 min on a rotary shaker, 10 \(\mu\)l of potassium acetate (2.5 M) and 10 \(\mu\)l of carrier-free \(^{22}\)NaCl (1 \(\mu\)Ci/\(\mu\)l) were added and the cells were allowed to metabolize for 60 min. Subsequently, 100-\(\mu\)l aliquots were withdrawn with a syringe.

To determine CO-dependent efflux of \(^{22}\)Na\(^+\), cells were incubated in the buffer described above containing 10 \(\mu\)Ci of \(^{22}\)Na\(^+\) for 18 h on ice. Subsequently, the reaction mixture was kept in darkness in a water bath at 37°C, and 20 mM potassium BES and 1 mM propyl iodide were added. The reaction was started by the addition of 1.6 ml of gaseous CO. Acetate-dependent efflux of \(^{22}\)Na\(^+\) was measured as follows. Cells were harvested and suspended in 3 ml of 20 mM imidazole buffer, pH 6.5, containing 20 mM MgCl\(_2\), 250 mM NaCl, and 5 mM dithioerythritol. A 100-\(\mu\)l portion of this cell suspension was incubated in the presence of 10 \(\mu\)Ci of \(^{22}\)Na\(^+\) in 2.3-ml serum tubes under N\(_2\) for 24 h on ice. At 20 min prior to zero time, the suspension was brought to 37°C and was diluted, at zero time, by the addition of 1 ml of 20 mM imidazole buffer, pH 6.5, containing 20 mM MgCl\(_2\), 250 mM sucrose, and 5 mM dithioerythritol. Potassium acetate was added to a final concentration of 50 mM. Determination of the radioactivity and preparation of the membrane filters were done as described previously (18).

**Chemicals and gases.** PIPES, DCCD, luciferin luciferase, and BES were purchased from Sigma, Taufkirchen, Federal Republic of Germany. TCS was from Eastman Kodak Co., Rochester, N.Y. \([7-^{14}\)C]benzoic acid, \([^{14}\)C]tetraphenylphosphonium bromide, \(^3\)H\(_2\)O, \([^{14}\)C]sucrose, and \(^{22}\)Na\(^+\) were purchased from New England Nuclear Corp., Dreieich, Federal Republic of Germany. The silicon oil was from Roth, Karlsruhe, Federal Republic of Germany. Gases were from Messer-Griesheim, Kassel, Federal Republic of Germany.

**RESULTS**

Generation of a proton motive force during methanogenesis from acetate and effect of DCCD. The following experiments were done with *M. barkeri* Fusaro that had been transferred in media with acetate as the sole carbon and energy source for more than 6 months. Cell suspensions of this strain formed methane from acetate under N\(_2\) at a maximal rate of 90 to 120 nmol/min per mg of protein. After a lag phase, this rate was constant for approximately 100 min. Methane formation was coupled to an increase in the intracellular ATP concentration to a level of 3.5 to 4.0 nmol/mg of protein. In the absence of acetate, the cells had an ATP content of only 0.8 to 0.9 nmol/mg of protein. H\(_2\) at concentrations of 0.5% in the gas phase completely blocked methanogenesis from acetate (data not shown).

The proton motive force (\(\Delta\psi\)) increased to a steady-state level of \(-120 \text{ mV}\) upon the addition of acetate. After termination of methane formation, this steady-state level did not decline rapidly but stayed at a high level for more than 3 h (Fig. 1). \(\Delta\psi\) consisted predominantly of the membrane potential (\(\Delta\psi\)); the transmembrane gradient of H\(^+\) (\(\Delta\text{pH}\)) was small and in the opposite orientation.

The addition of the uncoupler TCS to cell suspensions which were actively forming methane from acetate resulted in an inhibition of methane formation and in a rapid decrease in \(\Delta\psi\) (Fig. 2), as well as a rapid decrease in the intracellular ATP concentration (data not shown). DCCD, an inhibitor of proton-translocating ATPases of procaryotic organisms, including *M. barkeri* (10), also affected methanogenesis from acetate. The formation of methane stopped, and, in contrast to our expectation, \(\Delta\psi\) did not remain constant but decreased gradually (Fig. 2). This observation was different from

![FIG. 1. Magnitude and composition of \(\Delta\psi\) during methane formation from acetate by cell suspensions of *M. barkeri*. A 10-ml cell suspension (1.4 mg of protein per ml) was made in 100 mM sodium PIPES buffer, pH 6.7; 15 mM acetate was added at the time indicated by the arrow. Symbols: ▼, \(-62 \cdot \Delta\text{pH}; \) ■, \(\Delta\psi; \) ▲, \(\Delta\psi; \) ○, CH\(_4\).](http://jb.asm.org/Downloadedfrom/10.1128/JB.175.11.1370-1379.1993)

**FIG. 2. Effect of TCS and DCCD on methane formation from acetate and on \(\Delta\psi\). Experimental conditions were as described in the legend to Fig. 1. At the time indicated by the closed arrow, 15 mM acetate was added. TCS (final concentration, 10 \(\mu\)M) or DCCD (25 nmol/mg of protein) was added at the time indicated by the open arrow. Closed symbols, TCS experiment; open symbols, DCCD experiment; ○ and ○, CH\(_4\); ▲ and ▲, \(\Delta\psi\).](http://jb.asm.org/Downloadedfrom/10.1128/JB.175.11.1370-1379.1993)
observations made with *M. barkeri* forming methane from H₂ plus methanol (2) or from H₂ plus trimethylamine (19).

It was possible that the decline in Δp in the presence of DCCD was due to an uncoupling effect of acetate. Therefore, we examined whether acetate had an effect on Δp in acetate-grown cells which were energized with methanol plus H₂ and subsequently treated with DCCD (Fig. 3). A decline in Δp was not observed under these conditions.

**Role of Na⁺ in methanogenesis from acetate.** Possible reasons for the observed Na⁺ dependence of methane formation from acetate were investigated. *M. barkeri* cells were suspended in buffer containing various amounts of NaCl, 20 mM potassium BES, and 1 mM propyl iodide. The addition of gaseous CO to such suspensions led to H₂ formation irrespective of the external Na⁺ concentration (data not shown). This finding indicated that the CO dehydrogenase was functional in the absence of Na⁺ but did not exclude the possibility that at low external Na⁺ concentrations an uncoupling occurred between H₂ formation and ATP synthesis. Therefore, the effect of Na⁺ on the intracellular ATP content during methanogenesis from acetate and during H₂ formation from CO plus H₂O was investigated. In the presence of 0.7 mM NaCl, little methane was formed from acetate and the intracellular ATP level was low compared with that in the presence of 21 mM NaCl. Addition of Na⁺ to a final concentration of 25 mM to each suspension led to methane formation, as well as to net ATP synthesis (Fig. 4A). On the other hand, the same rate of ATP synthesis and the same intracellular ATP concentration were achieved when CO served as the energy source (Fig. 4B).

The intracellular sodium concentration during this fermentation was determined. Cells of *M. barkeri* were allowed to equilibrate in a buffer containing 250 mM ⁴²NaCl for 24 h on ice. During this period, the cells took up Na⁺ to a final concentration of 78 mM. At zero time, the suspension was diluted 10-fold by the addition of NaCl-free buffer, thus creating an outwardly directed Na⁺ gradient. The addition of acetate resulted in a decrease in the intracellular Na⁺ concentration (Fig. 5A). In the absence of a substrate, the intracellular Na⁺ concentration did not change. Correspondingly, cells incubated in the presence of 25 mM ⁴²NaCl extruded Na⁺ uphill against a concentration gradient upon the addition of CO (Fig. 5B). In a number of experiments, the magnitude of the Na⁺ gradient was determined during methanogenesis from acetate; it amounted to 4.0 ± 0.4 mM (Na⁺<sub>out</sub>/Na⁺<sub>in</sub>) at an external Na⁺ concentration of 25 mM. This transmembrane Na⁺ gradient was abolished by the addition of 10 µM TCS (data not shown).

**DISCUSSION**

In some anaerobic environments, acetate is the most important methanogenic substrate (6). However, the pathway of acetate conversion into methane and carbon dioxide is still not known in detail, and the bioenergetics of this conversion have not been studied until now.

Here, we showed that methanogenesis from acetate is accompanied by the generation of a proton motive force and an increase in the intracellular ATP level. The inhibitor studies with DCCD allowed us to conclude that with acetate as the methanogenic substrate, ATP is synthesized by a
chemiosmotic mechanism. Of note is the gradual decrease in Δp, which was not observed with the substrate combination methanol plus H₂ (2) but was observed with the combination CO₂ plus H₂ (3). In the case of methanogenesis from acetate, it may be explained as follows. After depletion of the ATP pool, acetate can no longer be activated. If Δp decreases as the result of transport or leakage processes, it cannot be replenished by methanogenesis because activated acetate, presumably acetyl coenzyme A, is not available. The decrease in Δp is not due to an uncoupling effect of acetate, since it did not occur with acetate plus methanol as the substrate.

The addition of the protonophore TCS resulted in an inhibition of methanogenesis and a decrease in both Δp and the intracellular ATP content. This is in contrast to methanogenesis with the substrate combination methanol plus H₂ (2) but is similar to that with methanol (4) and to that with H₂ plus CO₂ (3). This inhibition is conceivable if acetate must be activated prior to its conversion to CO₂ and methane.

Not yet understandable is the requirement of methanogenesis from acetate for a Na⁺ gradient. The Na⁺ gradient in M. barkeri has been shown not to be involved in pH regulation at acidic pH values or in ATP synthesis (4). Since this gradient is abolished by the proton conductor TCS, it is presumably the result of secondary Na⁺ translocation driven by the Na⁺/H⁺ antiporter, as has been shown for M. barkeri catabolizing methanol (18). From the pathway leading from acetate to methane and carbon dioxide the following reactions can be identified as functioning without a Na⁺ gradient: the conversion of acetate to acetyl coenzyme A, as catalyzed by the enzymes acetate kinase and phosphotransacetylase (12, 15); the reduction of methyl coenzyme M, which was recently identified as an intermediate in methanogenesis from acetate (16); ATP synthase (4); and the CO₂ dehydrogenase (reference 5 and this study). However, at least two reactions remain that could be driven by a sodium motive force: the uptake of acetate and the cleavage of acetyl coenzyme A. The latter reaction is endergonic, with a ΔG⁰⁺ of +40.3 kJ per reaction (11). An electrochemical Na⁺ potential could drive this reaction as it drives the oxidation of methanol to the level of formaldehyde in M. barkeri (V. Müller, unpublished results). Such a conclusion is very tempting because it readily explains why it has been very difficult to develop a cell-free system that catalyzes the conversion of acetyl coenzyme A to methane and carbon dioxide. Baresi (1) reported that this is possible only with pelleted membrane fractions. In another system, H₂ was required (14) which was shown to inhibit methane formation from acetate in M. barkeri Fusaro (7). It is not yet apparent why it is advantageous to M. barkeri (and possibly to other methanogenic bacteria) to use a proton motive force for ATP synthesis and a sodium motive force for certain reactions involved in methane formation from methanol, CO₂ plus H₂, or acetate.

ACKNOWLEDGMENTS

We are indebted to the staff of the Zentrales Isotenplab mit der Universität Göttingen for their helpful advice in performing the ²³Na experiments. The skilful technical assistance of K. Freisil is gratefully acknowledged. We are indebted to G. Kozianowski for assistance.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

LITERATURE CITED

10. Inatomi, K. I. 1986. Characterization and purification of the membrane-bound ATPase of the archaeabacterium Methanosar-

Received from http://jb.asm.org/ Downloaded on May 2, 2016 by guest