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Methanogenesis from trimethylamine + H_2 by *Methanosarcina barkeri* is coupled to ATP formation by a chemiosmotic mechanism

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Cell suspensions of *Methanosarcina barkeri* catalyzed the conversion of trimethylamine and molecular hydrogen to methane according to the equation $(CH_3)_3NH^+ + 3\ H_2 \rightarrow 3\ CH_4 + NH_4^+$. The onset of methane formation resulted in an increase of the intracellular ATP content from 2 to 4.6 nmol/mg protein and in the generation of a protonmotive force (Δp) of -130 mV, of which the $\Delta\psi$ contributed 90%. The addition of the uncoupler led to a drastic decrease of the intracellular ATP content and the $\Delta\psi$, but stimulated methanogenesis. The ATPase inhibitor DCCD caused a rapid exhaustion of the ATP pool and inhibited methane formation, whereas $\Delta\psi$ was not affected. The inhibition of methane formation by DCCD could be relieved by addition of TCS, indicating a chemiosmotic coupling between methane formation according to the above equation and ATP synthesis.

Introduction

Methanogenic bacteria are a phylogenetically diverse but nutritionally rather uniform group of strictly anaerobic organisms. Most species are able to grow on $H_2 + CO_2$ as a source of energy and carbon. Of all methanogens isolated so far, *Methanosarcina barkeri* is the metabolically most versatile organism: besides $H_2 + CO_2$ it can utilize methanol, acetate [1], methanol + H_2 [2], CO [3], methyl- dimethyl- and trimethylamine [4].

For a long time, the coupling mechanism between methane formation from all these substrates and ATP synthesis was unknown. Most investigations on the energy metabolism of methanogenic bacteria were done with *Methanobacterium thermoautotrophicum* using $H_2 + CO_2$ as methanogenic substrate [5–9]. Since this pathway is rather complex and involves several novel coenzymes, and carbon at four different oxidation states, we studied in our laboratory the energy conservation in resting cells of *M. barkeri* during methanogenesis from methanol + H_2 or formaldehyde + H_2 [10–12]. Using these substrates it was shown, that methane formation was coupled to ATP formation by a chemiosmotic mechanism. This conclusion was based on the following findings: (i) Addition of the uncoupler TCS to a cell suspension forming methane from the above-mentioned substrates resulted in a dissipation of $\Delta\psi$ and a decrease in the

Abbreviations: TCS, 3,5,4′,5′-tetrachlorosalicylanilide (3,5-dichloro-*N*-(4,5-dichlorophenyl)-2-hydroxybenzamide); DCCD, *N*,*N*′-dicyclohexylcarbodiimide; ΔpH, transmembrane chemical gradient of H^+; $\Delta\psi$, transmembrane electrical gradient, Δp, protonmotive force (= $\Delta\psi - 62$ mV·ΔpH); Ph_4PBr, tetraphenylphosphonium bromide; Tes, *N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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intracellular ATP content, whereas methane formation was stimulated. (ii) Addition of the ATPase inhibitor DCCD to such suspensions prevented ATP formation and inhibited methane formation but left $\Delta\psi$ intact. (iii) Addition of TCS to a cell suspension incubated in the presence of DCCD restored the ability to form methane. Interestingly, methane formation from $H_2 + CO_2$ or methanol alone was inhibited by TCS, indicating a strong coupling between exergonic and endergonic reactions during methane formation from these substrates.

The aim of this work was to study the effect of TCS and DCCD on Δp, intracellular ATP content and methane formation from trimethylamine $+ H_2$. The results presented clearly indicate a chemiosmotic coupling between ATP synthesis and methane formation from trimethylamine $+ H_2$ by *M. barkeri*.

Materials and Methods

Organism and culture condition. Methanosarcina barkeri, strain Fusaro (DSM 804) was grown on trimethylamine (final concentration, 100 mM) in 1200 ml glass bottles filled with 500 ml of the medium described by Hippe et al. [4] under an atmosphere of N_2/CO_2 (80 : 20). The pH was 6.5 to 6.8 and the growth temperature was 37 °C. The anaerobic techniques for medium preparation and for cultivation were those of Hungate [13] as modified by Bryant [14].

Preparation of cell suspensions. Fresh cell suspensions of *M. barkeri* were prepared for each experiment. Cells of the late logarithmic growth phase were harvested by centrifugation, washed once with 100 mM Pipes-NaOH buffer (pH 6.8) containing per liter 1 mg resazurin and 2 ml titanium(III) citrate solution [15]. The cell suspension was stored on ice until used. All manipulations were done under strictly anaerobic conditions in an anaerobic glove box (Mecaplex, Grenchen, Switzerland). The protein content was determined according to Ref. 16.

The experiments were carried out in 58 ml bottles containing 9.0 to 9.5 ml Pipes-NaOH buffer. The bottles were flushed with hydrogen, and 0.5 to 1.0 ml of the concentrated cell suspension was added anaerobically to the buffer to give a final protein concentration of 1 to 2 mg/ml. The resulting cell suspension was preincubated for 15 min at 37 °C on a rotary shaker. Additions were made anaerobically by syringe; trimethylamine was added as aqueous solution whereas TCS and DCCD were added as ethanolic solutions.

Determination of methane. Samples of 5 µl were taken from the gas phase for determination of methane by gas chromatography as described previously [17].

Determination of ATP. The ATP content of the cells was determined using the luciferin-luciferase assay as described previously [10]. Samples (0.5 ml) of the cell suspension were withdrawn by syringe, transferred directly into 0.2 ml ice-cold 3 M perchloric acid and kept on ice for 2 h. The pH was then adjusted to 7.4 by the addition of 65 µl of a saturated K_2CO_3 solution and 0.1 ml of 0.4 M Tes-NaOH buffer. The $KClO_3$ formed was removed by centrifugation. The supernatants were kept on ice until ATP determination. 20 µl of the supernatants were transferred into 1 ml of the assay buffer described by Kimmich et al. [18]. The reaction was started by addition of 50 µl of a luciferin-luciferase preparation and light flashes were determined in a liquid scintillation counter model LS 7500 (Beckmann, Fullerton, U.S.A.). Calibration was done using standards of known ATP-content.

Determination of $\Delta\psi$ and ΔpH. $\Delta\psi$ and ΔpH were estimated from the transmembrane equilibrium distribution of a lipophilic cation and a weak acid, respectively, according to Rottenberg [19]. For the determination of $\Delta\psi$, 1 µCi [^{14}C]Ph_4PBr was added to 10 ml of the resting cell suspension mentioned above to give a final Ph_4P^+ concentration of 10 µM. The internal and total water spaces of *M. barkeri* cells 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 .For the determination of ΔpH, 10 µCi ^3H_2O and 1 µCi [^{14}C]benzoic acid were added to 10 ml cell suspension. At the times indicated in the figures, 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 incubated

for 12 h in an anaerobic chamber. The cells were separated from the medium by centrifugation through the silicone oil. The supernatant and the pellet were assayed for ^{14}C and ^{3}H using a liquid scintillation counter model LS 7500 (Beckmann, Fullerton, U.S.A.). $\Delta\psi$, ΔpH and nonspecific binding of Ph_4P^+ was determined as described [10].

Chemicals. Pipes, *N,N'*-dicyclohexylcarbodiimide, luciferin-luciferase (FLE-250) were purchased from Sigma (Taufkirchen, F.R.G.), 3,5,4',5'-tetrachlorosalicylanilide was from Eastman Kodak (Rochester, U.S.A.). [7-^{14}C]Benzoic acid, [^{14}C]Ph_4PBr, $^{3}H_2O$ and [^{14}C]sucrose were purchased from New England Nuclear (Dreieich, F.R.G.). The silicone oil was from Roth (Karlsruhe, F.R.G.).

Results

Resting cells of *M. barkeri* grown on trimethylamine were incubated under H_2 at 37°C. At the time indicated by the arrow, trimethylamine was added to the suspension and the cells produced

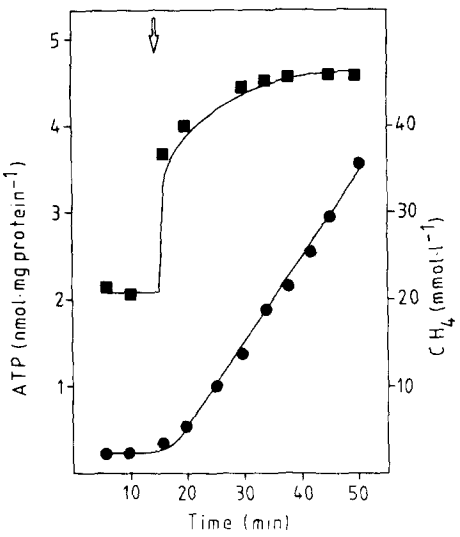

Fig. 1. Methane and ATP formation during conversion of trimethylamine + H_2 by cell suspensions of *M. barkeri*. The incubation mixture contained 100 mM Na-Pipes (pH 6.8) and 1.1 mg protein/ml under H_2. Trimethylamine (final concentration 20 mM) was added as indicated by the arrow. CH_4 (●); ATP (■).

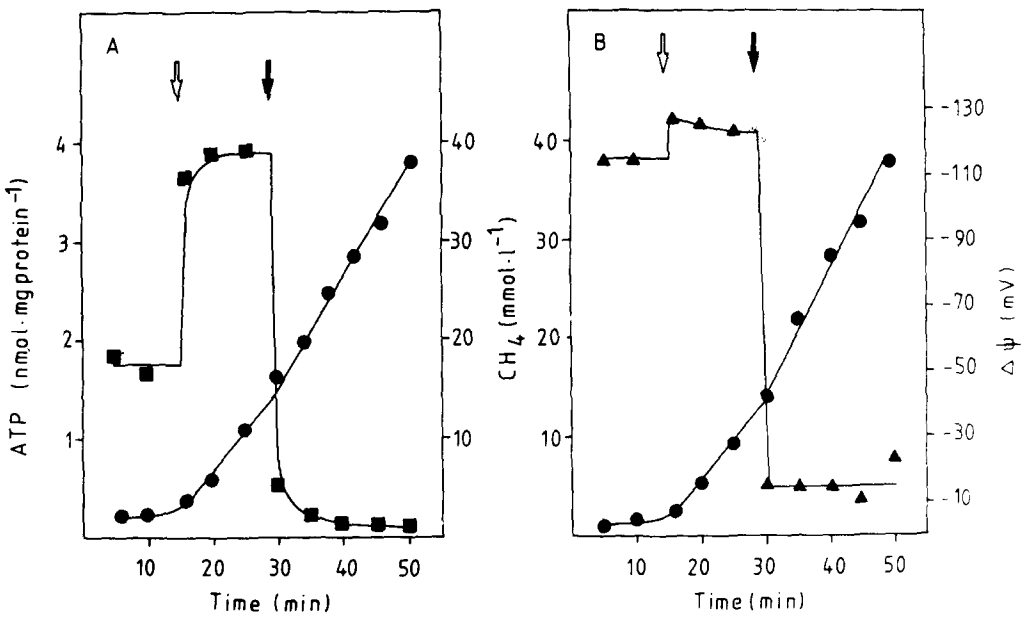

Fig. 2. Effect of TCS on methanogenesis, $\Delta\psi$ and ATP. (A) methane formation and intracellular ATP content, (B) methane formation and $\Delta\psi$. The incubation mixture contained 100 mM Na-Pipes (pH 6.8) and 1.1 mg protein/ml under H_2. Trimethylamine was added (open arrow) to a final concentration of 20 mM, TCS (closed arrow) to a final concentration of 10 μM. ATP (■); CH_4 (●); $\Delta\psi$ (▲).

methane with a linear rate of 0.4 μmol CH_4/min per mg protein. The onset of methane formation was accompanied by an increase in the intracellular ATP concentration which reached a plateau of about 4.6 nmol ATP/mg protein (Fig. 1). Correspondingly, Δp increased upon trimethylamine addition to a steady state level of −130 mV. The Δp consisted predominantly of the $\Delta\psi$, and ΔpH contributed only to a negligible amount to the Δp (data not shown). Therefore, only $\Delta\psi$ was measured in the following experiments. Labeling studies revealed that in the presence of H_2 trimethylamine was exclusively reduced to methane; CO_2 was not formed from it (data not shown).

Addition of the uncoupler, TCS, to cell suspensions actively forming methane from trimethylamine + H_2 led to a slight increase of the methane formation rate. However, the intracellular ATP concentration and $\Delta\psi$ decreased (Fig. 2). In a separate experiment, in which no liquid samples but more samples for the determination of methane were taken, the stimulation of methane formation

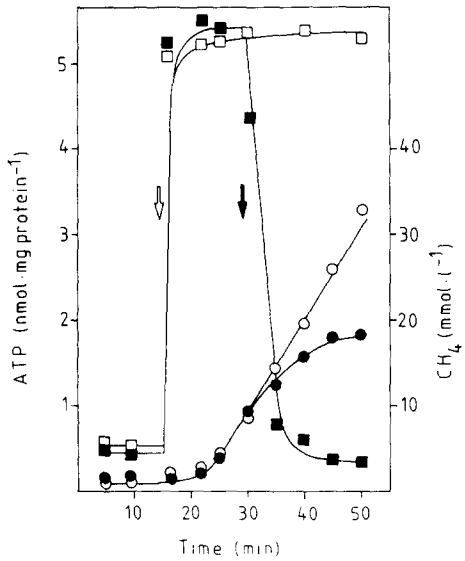

Fig. 3. Effect of DCCD on methane formation from trimethylamine and H_2 and on the intracellular ATP content. The incubation mixture contained 100 mM Na-Pipes (pH 6.8) and 1.0 mg protein/ml under H_2. Trimethylamine was added (open arrow) to a final concentration of 20 mM, DCCD (closed arrow) to a final concentration of 30 nmol/mg protein. CH_4 (○) and ATP (□) in the absence of DCCD; CH_4 (●) and ATP (■) in the presence of DCCD.

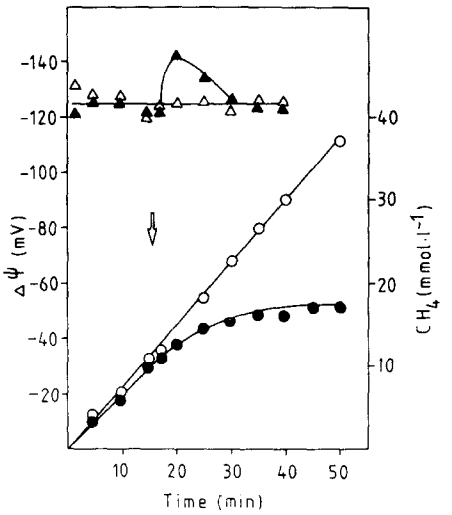

Fig. 4. Effect of DCCD on methane formation from trimethylamine and H_2 and on $\Delta\psi$. The incubation mixture contained 100 mM Na-Pipes (pH 6.8) and 1.1 mg protein/ml under H_2. The reaction was started by addition of trimethylamine (final concentration: 30 mM) to the cell suspension 30 min prior to zero time. The CH_4 produced at zero time was 25 mmol/l. DCCD was added as indicated by the open arrow to a final concentration of 30 nmol/mg protein. CH_4 (○) and $\Delta\psi$ (△) in the absence of DCCD, CH_4 (●) and $\Delta\psi$ (▲) in the presence of DCCD.

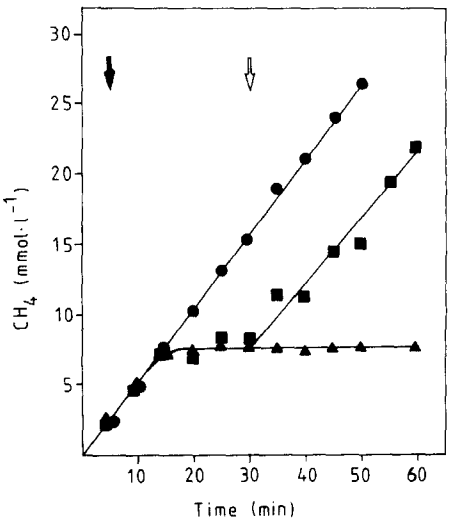

Fig. 5. Effect of TCS on methanogenesis from trimethylamine + H_2 by cells previously inhibited by DCCD. The incubation mixture contained 100 mM Na-Pipes (pH 6.8), and 1.0 mg protein/ml under H_2. Trimethylamine was added to a final concentration of 20 mM at zero time, DCCD (final concentration: 30 nmol/mg protein) as indicated by the closed arrow, TCS (final concentration: 10 μM) as indicated by the open arrow. No further additions (●), addition of DCCD (■, ▲), addition of DCCD followed by addition of TCS (■).

DCCD is known to inhibit membrane-bound protontranslocating ATPases in mitochondria, eubacteria and archaebacteria [20–23]. The addition of DCCD to an actively metabolizing cell suspension of *M. barkeri* forming methane from trimethylamine + H_2 led to a decrease in the methane formation rate. At the same time, the intracellular ATP concentration decreased (Fig. 3). In contrast, $\Delta\psi$ temporarily increased after the addition of DCCD and remained then at the same level as in the absence of DCCD (Fig. 4).

The inhibition of methane formation by DCCD could be relieved by the addition of TCS (Fig. 5), suggesting a strong coupling between proton translocation and methane formation from trimethylamine + H_2.

Discussion

M. barkeri is able to grow on mono-, di- or trimethylamine [4]. The methyl group of trimethylamine is transferred to mercaptoethanesulfonate by a specific methyltransferase [24]. The 2-(methylthio)ethanesulfonate is then reductively cleaved to yield methane and 2-mercaptoethanesulfonate [25,26]. Therefore, it is reasonable that the results obtained with trimethylamine + H_2 are very similar to those previously obtained with methanol + H_2. With both substrate combinations 2-(methylthio)ethanesulfonate is formed in methyltransferase reactions [24,27], and the reducing equivalents for the terminal reaction are provided by H_2. The effects of TCS and DCCD on methanogenesis, $\Delta\psi$ and the ATP level were analogous and strengthened the conclusion previously drawn that ATP is synthesized by a chemiosmotic mechanism and that the methylreductase system is involved in proton translocation. At least for one methanogenic organism (*Methanococcus voltae*) it has now been demonstrated by immunolabeling that component C of the methylreductase system is indeed membrane-associated [28].

The clear-cut effect of TCS on the level of ATP and $\Delta\psi$ in *M. barkeri* as observed during methanogenesis with methanol + H_2 [10], with formaldehyde + H_2 [11] and with trimethylamine + H_2 or during conversion of CO to CO_2 + H_2 [29] is in contrast to results reported for *M. thermoautotrophicum* and *M. voltae*. In these organisms a continuation of ATP synthesis during methanogenesis from H_2 + CO_2 in the presence of an uncoupler was observed [9,30]. At least in *M. thermoautotrophicum*, this effect was only seen at low concentrations of the uncoupler while higher amounts inhibited methane formation as well as ATP synthesis [9]. A reasonable explanation for these findings cannot be given at the moment. From our point of view, studies on the bioenergetics of methanogenesis should concentrate first on those methane forming pathways which include only exergonic steps: the substrate combinations methanol + H_2, formaldehyde + H_2 and trimethylamine + H_2 have this in common.

The effect of DCCD on *M. barkeri* is comparable to that on mitochondria [31]. This is not true for all methanogenic bacteria. In some of them DCCD fails to inhibit methanogenesis [9,32–34]. The effectiveness of DCCD might be related to differences in cell wall composition. In this connection results on a new methanogenic isolate are interesting: strain AJ 2, which is surrounded by a proteinaceous cell envelope, was shown not to be DCCD-sensitive. However, methanogenesis from methanol + H_2 by protoplasts of this microorganism was affected by DCCD [35].

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