Methanogenesis from Acetate by *Methanosarcina barkeri*: Catalysis of Acetate Formation from Methyl Iodide, CO_2, and H_2 by the Enzyme System Involved*

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* *Dedicated to Professor Helmut Simon on the occasion of his 60th birthday*

Methanogenesis from Acetate, Acetyl-CoA as Intermediate, Carbon Monoxide Dehydrogenase, Corrinoid Enzymes, *Methanosarcina barkeri*

Cell suspensions of *Methanosarcina barkeri* grown on acetate catalyze the formation of methane and CO_2 from acetate as well as an isotopic exchange between the carboxyl group of acetate and CO_2. Here we report that these cells also mediate the synthesis of acetate from methyl iodide, CO_2, and reducing equivalents (H_2 or CO), the methyl group of acetate being derived from methyl iodide and the carboxyl group from CO_2. Methyl chloride and methyltosylate but not methanol can substitute for methyl iodide in this reaction. Acetate formation from methyl iodide, CO_2, and reducing equivalents is coupled with the phosphorylation of ADP. Evidence is presented that methyl iodide is incorporated into the methyl group of acetate *via* a methyl corrinoid intermediate (deduced from inhibition experiments with propyl iodide) and that CO_2 is assimilated into the carboxyl group *via* a C_1 intermediate which does not exchange with free formate or free CO. The effects of protonophores, of the proton-translocating ATPase inhibitor N,N′-dicyclohexylcarbodiimide, and of arsenate on acetate formation are interpreted to indicate that the reduction of CO_2 to the oxidation level of the carboxyl group of acetate requires the presence of an electrochemical proton potential and that acetyl-CoA or acetyl-phosphate rather than free acetate is the immediate product of the condensation reaction. These results are discussed with respect to the mechanism of methanogenesis from acetate.

Introduction

Methanosarcina barkeri is a methanogenic bacterium that can grow on acetate as sole energy source [1–3] (reaction (a)).

$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$$
$$\Delta G'_o = -36 \text{ kJ/mol} \quad \text{(a)}$$

Methane is derived from the methyl group and CO_2 from the carboxyl group of acetate [4, 5]. Trideutero-acetate yields trideuteromethane [6–8]. Available evidence indicates that methanogenesis from acetate involves the following partial reactions (b–h):

$$CH_3COO^- + H^+ + ATP \rightleftharpoons CH_3CO-P + ADP \quad \text{(b)}$$

$$CH_3CO-P + CoA \rightleftharpoons CH_3CO-CoA + P_i \quad \text{(c)}$$

$$CH_3CO-CoA + X + Y \rightleftharpoons CH_3X + CO-Y + CoA \quad \text{(d)}$$

$$CO-Y + H_2O \rightleftharpoons CO_2 + Y + 2[H] \quad \text{(e)}$$

$$CH_3X + [Co]E \rightarrow CH_3[Co]E + X \quad \text{(f)}$$

$$CH_3[Co]E + CoM \rightarrow CH_3CoM + [Co]E \quad \text{(g)}$$

$$CH_3CoM + 2[H] \rightarrow CH_4 + CoM. \quad \text{(h)}$$

Acetyl-phosphate and acetyl-CoA are considered to be intermediates (reactions (b) and (c)) [9] because acetate kinase (EC 2.7.2.1) and phosphotransacetylase (EC 2.3.1.8) are induced upon growth of the bacterium on acetate [10]. Also, in a cell-free system, acetyl-phosphate rather than acetate is converted to methane and CO_2 [11]. Acetyl-CoA is cleaved to CH_3X, CO–Y, and CoA [12] (reaction (d)) probably by the action of carbon monoxide dehydrogenase [11]. X is most certainly a transition metal, however, it is still controversial whether it is the nickel of carbon monoxide dehydrogenase [13, 14] or a cobalt of a corrinoid enzyme [15, 16].

Y to which a C_1 unit at the oxidation level of CO is bound [17] is considered to be carbon monoxide dehydrogenase which is assumed to catalyze the oxidation of CO–Y to CO_2 [18] (reaction (e)) although no direct evidence for this is presently available.

Abbreviations: CoM, 2-mercaptoethanesulfonic acid; CH_3CoM, 2-methylthioethanesulfonic acid; methyltosylate, methyl 4-toluenesulfonate; DCCD, N,N′-dicyclohexylcarbodiimide; TCS, 3,5,3′,4′-tetrachlorosalicylanilide; Ph_4P^+, tetraphenylphosphonium cation; $\Delta\Psi = -R\mathrm{Tln}([Ph_4P^+]_{in}/[Ph_4P^+]_{out})/F$, transmembrane electrical gradient; $\Delta\tilde{\mu}_H+$, electrochemical proton potential.

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Inhibition studies with propyl iodide indicate that the methyl group is transferred from X to a corrinoid enzyme [12] (reaction (f)) and from there to CoM [8] (reaction (g)). Methyl-CoM reduction to methane is catalyzed by CH_3CoM reductase [19–21], which contains a nickel porphinoid as prosthetic group [22–27] (reaction (h)).

It has been demonstrated that both the oxidation of CO–Y to CO_2 (reaction (e)) [18] and the reduction of CH_3CoM to methane [28] (reaction (h)) are coupled with the phosphorylation of ADP *via* a chemiosmotic mechanism.

The reversibility of reactions (b–e) is deduced from the finding that acetate grown cells mediate a rapid exchange between the carboxyl group of acetate and CO_2 [17]. Reaction (g) is considered to proceed irreversibly since methanol, which is rapidly converted to CH_3CoM by the cells, is not incorporated into the methyl group of acetate [12]. Whether reaction (f) is reversible or not is not known.

In this communication we report that acetate grown cells of *M. barkeri* catalyze the formation of acetate from methyl iodide, CO_2, and H_2 in a reaction coupled with the phosphorylation of ADP. The results indicate that CH_3I reacts with the methyl acceptor X (in reaction (d)) to yield CH_3X and I^- (reaction (i)).

$$CH_3I + X^- \rightarrow CH_3X + I^- \qquad \text{(i)}$$

This reaction is irreversible since no exchange between CH_3I and the methyl group of acetate was observed. CH_3X thus formed can react with CO_2 and reducing equivalents to yield acetate *via* the reversible reactions (b–e). The reducing equivalents are provided in reaction (j), which is catalyzed by hydrogenase present in *M. barkeri* [29].

$$H_2 \rightarrow 2H^+ + 2e^- \qquad \text{(j)}$$

Materials and Methods

Chemicals and bacteria

3,5,3′,4′-Tetrachlorosalicylanilide (TCS) was a gift from Eastman Kodak Co. (Rochester, USA). N,N′-Dicyclohexylcarbodiimide (DCCD) and immersion oil for microscopy were from Merck (Darmstadt, FRG). Methyltosylate was from Fluka (Buchs, Switzerland). Tetra[U-^{14}C]phenylphosphonium bromide ([^{14}C]Ph_4P^+), [^{14}C]CH_3I, and [^{14}C]Na_2CO_3 were obtained from Amersham Buchler (Braunschweig, FRG). [^{14}C]HCOONa, [1-^{14}C]CH_3COONa, and [2-^{14}C]CH_3COONa were from New England Nuclear (Dreieich, FRG). ^{14}CO was prepared from [^{14}C]formate [30]. ATP-monitoring reagent (lyophilized mixture of firefly luciferase and luciferin) was from LKB Instrument GmbH (Gräfelfing, FRG). *Methanosarcina barkeri* strain Fusaro (DSM 804) was from the Deutsche Sammlung von Mikroorganismen (Göttingen, FRG).

Growth of bacteria

M. barkeri [31] was grown in the dark at 37 °C in 1 l bottles (gas phase 100% N_2) in 400 ml medium containing 100 mM sodium acetate as sole carbon and energy source [32]. The medium (initial pH 6.4) was inoculated with 5% of a late-log-phase culture. Growth was followed by measuring methane formation and the increase of protein. The pH was maintained between 6.6 and 7.0 by the addition of glacial acetic acid. The cells grew within 5 days to a cell concentration of approximately 125 mg protein/l at which the cell suspensions were prepared.

Preparation of cell suspensions

Samples (30–60 ml) from the 400 ml culture of *M. barkeri* were transferred anaerobically into 120 ml serum bottles closed with rubber stoppers and filled with 100% N_2. The cells were sedimented by centrifugation at $3000 \times g$ for 20 min at 4 °C and the supernatant was discarded. The cells were then washed twice with anaerobic imidazole phosphate suspension buffer (20 mM NaH_2PO_4, 5 mM KH_2PO_4, 20 mM imidazole adjusted to pH 7.4 with KOH, 2 mM $MgCl_2$, 40 mM NaCl, 5 mM dithiothreitol, and 20 μM resazurin), and suspended in 3–6 ml of this buffer to give a final protein concentration of approximately 1 mg per ml. When the effect of arsenate was studied the imidazole phosphate suspension buffer was replaced by a 45 mM potassium morpholinopropanesulfonate buffer pH 7.4 containing 10 mM $MgCl_2$, 0.1 mM $(NH_4)Fe(SO_4)_2$, 5 mM dithiothreitol, and 20 μM resazurin. For determination of protein, samples (200 μl) of the cell suspensions were mixed with 300 μl 0.3 M NaOH and then heated to 100 °C for 90 min. Protein in the partial hydrolysates was quantitated by the method of Bradford [33].

Experiments with cell suspensions

The cell suspension in the 120 ml serum bottles was generally transferred anaerobically into sealed

25 ml serum bottles in which the assays were performed (where indicated the experiments were performed in the 120 ml serum bottles). The gas phase was 80% N_2, 20% CO_2, and 0.6% H_2 or 75% N_2, 19% CO_2, and 6% CO as indicated. Due to the CO_2 in the gas phase the pH of the suspensions decreased from 7.4 to 7.0. Substrates and inhibitors were added anaerobically by syringes at concentrations, combinations, and specific radioactivities as described in the tables and figures. $^{14}CO_2$ was added as $[^{14}C]Na_2CO_3$ and allowed to equilibrate with $^{12}CO_2$ in the gas phase. Unless otherwise stated the assays were started by increasing the temperature from 0 °C to 37 °C. Incubation was in the dark in a gyrotory water-bath shaker at 200 rpm. At the times indicated samples of the gas phase (0.3 ml) and of the liquid phase (0.5 ml) were withdrawn with syringes. The gas phase was analyzed for CH_4, CO_2, H_2, and CO and the liquid phase for acetate, ATP, and $\Delta\Psi$.

Determination of gases

CO [17], CO_2 [17], H_2 [34], and CH_4 [34] were quantified by gas chromatography.

Determination of acetate

Acetate was determined enzymatically with acetyl-CoA synthetase [35]. For the isolation of [^{14}C]acetate, samples of the cell suspension were acidified with $HClO_4$ and shaken for 3 h to remove the $^{14}CO_2$. After neutralization with KOH the cell suspensions were centrifuged for 2 min at $10{,}000 \times g$. Then acetate was isolated from the supernatant by chromatography on Dowex 1×8 (100–200 mesh), formate form [36]. For the determination of the label pattern acetate was subjected to Schmidt degradation as described by Simon and Floss [37]. C1 of acetate was released as CO_2 which was trapped in 1 M NaOH and then counted for radioactivity. Methylamine (= C2 of acetate) was oxidized by $KMnO_4$, the CO_2 formed was trapped in NaOH, and counted for radioactivity.

Determination of specific radioactivities of CO_2 and CO

The specific radioactivity of CO_2 in the gas phase was determined by measuring the concentration of CO_2 gas chromatographically and the radioactivity after absorption in NaOH by counting in Aqualuma®. The specific radioactivity of CO was determined after oxidation to CO_2 with palladium chloride [38].

Determination of ATP

The ATP content of the cells was determined using the luciferin/luciferase assay as described by Schönheit and Beimborn [39]. Samples (0.5 ml) of the cell suspension were transferred directly into 1 ml ethanol at −20 °C and rapidly mixed. The mixture was maintained for 2 h in an ice/salt bath at −20 °C, then flash-evaporated at 50 °C to dryness, and the residue was dissolved in 0.5 ml 20 mM Tris/HCl buffer, pH 7.5, containing 0.2 mM EDTA (ethylenediaminetetraacetic acid, Titriplex III), 0.05 mM dithiothreitol, 0.5 mM $Mg(CH_3COO)_2$, and 0.5% bovine serum albumin. Aliquots of 25 µl were immediately analyzed for ATP in 500 µl Tris/acetate buffer with 10 µl "ATP-monitoring reagent" (125 mg luciferin/luciferase mixture dissolved in 10 ml H_2O) using a Lumac Biocounter M 2000 (Abimed, Düsseldorf, FRG).

Determination of $\Delta\Psi$

$\Delta\Psi$ across the cytoplasmic membrane was determined by transmembrane equilibrium distribution of $[^{14}C]Ph_4P^+$ according to Rottenberg [40]. One µCi $[^{14}C]Ph_4P^+$ (31.4 Ci/mol) was added to the cell suspension in the serum bottles to give a final concentration of 3.2 µM Ph_4P^+. At the times indicated in the figures samples (0.5 ml) of the cell suspension were transferred to 1.5 ml microfuge tubes containing 0.2 ml immersion oil (ϱ = 1.02 g·cm^{-3}), which had been incubated for at least 12 h in an anaerobic chamber. The cells were separated from the medium by centrifugation through the immersion oil in a microfuge. The supernatant and the cell pellet were analyzed for ^{14}C, $\Delta\Psi$ was calculated from the radioactivity distribution as previously described for *M. barkeri* by Blaut and Gottschalk [41].

Results

Cell suspensions of acetate grown *Methanosarcina barkeri* catalyzed the following reactions at rates given in parentheses in nmol·min^{-1}·mg protein^{-1}: methane formation from acetate (70–150), from H_2 and CO_2 (80–120), from H_2 and methanol (20–40), from methanol (20–40), and an isotopic exchange between CO_2 and the carboxyl group of acetate

(70–150). We describe here that the cells also catalyzed the synthesis of acetate from CH_3I, CO_2, and reducing equivalents (20–40). (For conditions see the legends to Fig. 1 and 2.)

Methanogenesis from acetate, the CO_2/acetate exchange reaction, and acetate synthesis from CH_3I, CO_2, and reducing equivalents were only observed with acetate grown cells of *M. barkeri*. Cells grown on H_2 and CO_2 or on methanol were devoid of these activities (< 1 nmol · min^{-1} · mg protein^{-1}). As a working hypothesis it is therefore assumed that methanogenesis from acetate, the CO_2/acetate exchange reaction, and the synthesis of acetate from CH_3I, CO_2, and reducing equivalents involve common enzymes and partial reactions.

In the following the effect of CH_3I on methanogenesis from various substrates and on the CO_2/acetate exchange reaction is described. Then the synthesis of acetate from CH_3I, CO_2, and reducing equivalents is characterized with respect to the kinetics, energetics, and mechanism.

The effect of CH_3I on methanogenesis and on the CO_2/acetate exchange reaction

Methyl iodide at a concentration of 100 μM inhibited methanogenesis from acetate, from H_2 plus CO_2, from H_2 plus methanol, and from methanol. At this concentration the CO_2/acetate exchange activity was not affected (Fig. 1). An inhibitory effect of CH_3I on the exchange activity was observed only at concentrations higher than 100 μM. In the presence of 2 mM CH_3I the specific rate of CO_2/acetate exchange was still 30% (20–40 nmol · min^{-1} · mg protein^{-1}) of that observed in the absence of CH_3I (70–150 nmol · min^{-1} · mg protein^{-1}). At a concentration of 2 mM CH_3I the specific rate of acetate formation from CH_3I, CO_2, and H_2 was 20–40 nmol · min^{-1} · mg protein^{-1}.

Conclusions: from these results three conclusions can be drawn: (i) at low concentrations (100 μM) CH_3I has no effect on reactions (b–e), which are involved in CO_2/acetate exchange; (ii) acetate formation from CH_3I, CO_2, and H_2 and the CO_2/acetate exchange reaction are catalyzed by the same enzyme system. This is concluded from the finding that at high CH_3I concentrations (2 mM) the rate of acetate formation from CH_3I, CO_2, and H_2 and of the CO_2/acetate exchange reaction were almost identical; (iii) CH_3I (100 μM) inhibits a reaction, which is common

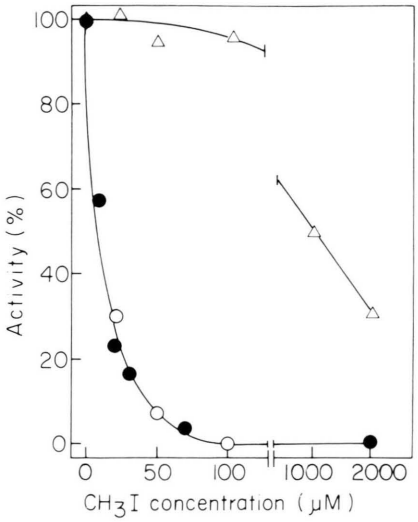

Fig. 1. Effect of CH_3I on reactions catalyzed by cells of *Methanosarcina barkeri*.
(●) Methane formation from acetate (100% = 125 nmol · min^{-1} · mg protein^{-1} of acetate grown cells);
(○) methane formation from H_2, CO_2, and methanol (100% = 225 nmol · min^{-1} · mg protein^{-1} of methanol grown cells).
(△) Isotopic exchange between CO_2 and the carboxyl group of acetate (100% = 125 nmol · min^{-1} · mg protein^{-1} of acetate grown cells).
The assays were performed in sealed 25 ml serum bottles containing 3 ml cell suspension (1.3 mg protein per ml). The gas phase was 80% N_2 (or 80% H_2) and 20% CO_2 (or 20% $^{14}CO_2$) at 120 kPa pressure. The concentrations of acetate and of methanol were 50 mM. The activities were determined after 30 min.

to methanogenesis from acetate, from CO_2, and from methanol, *i.e.* the CH_3CoM reductase reaction [42] (reaction (h)). Therefore, the CH_3CoM reductase cannot be involved in either the CO_2/acetate exchange reaction or the synthesis of acetate from CH_3I, CO_2, and H_2.

Synthesis of acetate from CH_3I, CO_2, and H_2

Acetate grown cells of *M. barkeri* catalyzed the formation of acetate from CH_3I, CO_2, and H_2 linearly with time up to 10 min (see Fig. 2) and with protein concentration up to 2–3 mg per ml. The apparent K_m values for CH_3I, CO_2, and H_2 were 0.5 mM, 10% and 0.2% in the gas phase, respectively. Maximal rates (20–40 nmol · min^{-1} · mg protein^{-1}) were achieved at 2–4 mM CH_3I, 20–40% CO_2, and

0.5–1.5% H_2. Higher concentrations of CH_3I and of H_2 proved inhibitory.

In the absence of H_2 the rate of acetate formation from CH_3I (2 mM) and CO_2 (20%) was 15 nmol · min^{-1} · mg $protein^{-1}$. Under these conditions $^{14}CH_3I$ was oxidized to $^{14}CO_2$ and thus served both as methyl group donor and as electron donor in acetate formation.

Acetate grown cells also mediated the formation of acetate from methyltosylate (3 mM), CO_2 (20%), and H_2 (0.5%) (V_{max} = 25–35 nmol · min^{-1} · mg $protein^{-1}$), and from methyl chloride (10% in the gas phase), CO_2 (20%), and H_2 (0.5%) (V_{max} = 20 nmol · min^{-1} · mg $protein^{-1}$). Methanol was unable to substitute for CH_3I.

Labelling experiments with $^{14}CH_3I$ plus CO_2 and with CH_3I plus $^{14}CO_2$ in the presence of H_2 showed that the methyl group of acetate derived from CH_3I and the carboxyl group from CO_2 (Table I).

Conclusions: from these results it is concluded that acetate formation proceeded according to reaction (k):

$$^{\bullet}CH_3I + {}^{\triangle}CO_2 + H_2 \rightarrow {}^{\bullet}CH_3{}^{\triangle}COO^- + 2H^+ + I^- \quad (k)$$

It was tested whether the cells catalyzed the reduction of CH_3I with H_2 to methane. Methane formation was not observed (< 1 nmol · min^{-1} · mg $protein^{-1}$), neither at low nor at high CH_3I concentrations (50 μM–2 mM). This can be explained by the finding that CH_3I is an inhibitor of the CH_3CoM reductase reaction (see above).

It was also examined whether the cells mediated an isotopic exchange between CH_3I and the methyl group of acetate. An incorporation of label from [2-^{14}C]acetate into CH_3I could not be detected. This finding indicates that methyl transfer from CH_3I to the methyl acceptor (reaction (i)) is an irreversible process.

Table I. Incorporation of ^{14}C into acetate from $^{14}CH_3I$, $^{14}CO_2$, ^{14}CO or $H^{14}COO^-$ during synthesis of acetate from CH_3I, CO_2, and reducing equivalents by cells of acetate grown *M. barkeri*. The assays were performed in sealed 25 ml serum bottles containing 4 ml cell suspension (0.5 mg protein per ml). The gas phase was 80% N_2, 20% CO_2, and 0.6% H_2 at 120 kPa pressure; in the experiment with ^{14}CO the gas phase was 75% N_2, 19% CO_2, and 6% ^{14}CO; in the experiment with $H^{14}COO^-$ the gas phase was 80% N_2 and 20% CO_2. The CH_3I concentration was always 2 mM. After 20 min 1 ml 3 M $HClO_4$ was injected, acetate was isolated and degraded as described in the methods section.

Added isotope[a]	Specific radioactivity of acetate C1+2	acetate C1 (Bq/μmol)	acetate C2
$^{14}CH_3I$	3000	460	2340
$^{14}CO_2$	3000	2600	3
^{14}CO[b]	65	n.d.[c]	n.d.
$H^{14}COO^-$ (10 mM)[d]	3	n.d.	n.d.

[a] Specific radioactivity of [^{14}C]compounds = 3000 Bq/μmol.
[b] Due to the oxidation of ^{14}CO to $^{14}CO_2$ the specific radioactivity of CO_2 increased from 0 to 80 Bq/μmol.
[c] n.d. = not determined.
[d] $^{14}CO_2$ formation from $H^{14}COO^-$ was not observed.

Synthesis of acetate from CH_3I, CO_2, and CO

Acetate grown cells of *M. barkeri* mediated the formation of acetate from CH_3I (2 mM), CO_2 (19%), and CO (6%) at a maximal rate of 20–40 nmol · min^{-1} · mg $protein^{-1}$. The apparent K_m value for CO was determined to be 2% CO in the gas phase. When the cells were incubated in the presence of ^{14}CO, $^{12}CO_2$, and CH_3I only very little radioactivity was incorporated into acetate (Table I). The CO_2 pool, however, became labelled. The cells mediated the oxidation of ^{14}CO to $^{14}CO_2$ at a specific rate of 80–120 nmol · min^{-1} · mg $protein^{-1}$. At the end of the experiment the specific radioactivities of CO_2 and of acetate were almost identical (Table I), indicating that ^{14}CO was incorporated into the carboxyl group of acetate *via* $^{14}CO_2$.

It was also tested whether formate could be used as an electron donor and/or carboxyl group precursor in the reaction. When the cells were incubated in the presence of [^{14}C]formate (2–10 mM), CO_2 (20%), and CH_3I (2 mM) neither [^{14}C]acetate nor $^{14}CO_2$ were formed. Free formate can thus be excluded as an intermediate in acetate formation from CH_3I, CO_2, and reducing equivalents.

Conclusions: the results show that CO was used as electron donor rather than as direct carboxyl group precursor in acetate formation from CH_3I, CO_2, and CO (reaction (l)).

$$^{\bullet}CH_3I + {}^{\triangle}CO_2 + {}^{*}CO + H_2O \rightarrow {}^{\bullet}CH_3{}^{\triangle}COO^- + 2H^+ + I^- + {}^{*}CO_2 \quad (l)$$

Coupling of acetate synthesis with the phosphorylation of ADP

Synthesis of acetate from CH_3I, CO_2, and H_2 by acetate grown cells of *M. barkeri* was associated with the phosphorylation of ADP (Fig. 2). Upon start of

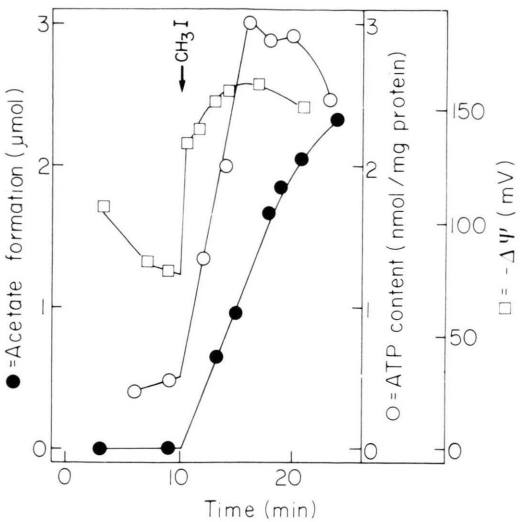

Fig. 2. Coupling of acetate formation from CH_3I, CO_2, and H_2 with the phosphorylation of ADP and the generation of $\Delta\Psi$ in cells of acetate grown *M. barkeri*. The assays were performed in sealed 25 ml serum bottles containing 6 ml cell suspension (1 mg protein per ml). The gas phase was 80% N_2, 20% CO_2, and 0.6% H_2 at 140 kPa pressure. The cells were incubated at 37 °C for 10 min before start of the reaction with CH_3I (2 mM). The ATP content and $\Delta\Psi$ were determined in separate experiments.

the reaction with CH_3I the ATP content in the cells increased from 0.5 nmol to 3 nmol per mg protein. An apparent stoichiometry of 0.01 mol ATP per mol acetate was observed. Acetate formation was also associated with a rapid increase in the membrane potential ($\Delta\Psi$) from 80 mV to 160 mV (inside negative). The rate of methanogenesis during acetate formation was less than 1 nmol · min^{-1} · mg protein^{-1}.

Conclusions: although the apparent stoichiometry between ADP phosphorylation and acetate formation was only very low we conclude from the results that acetate synthesis from CH_3I, CO_2, and H_2 is coupled with the synthesis of ATP. We found that in *M. barkeri* ATP is rapidly hydrolyzed *via* the membrane associated ATPase (results not shown). Upon inhibition of this enzyme by DCCD the apparent stoichiometry increased significantly to a value of 0.1 mol ATP per mol acetate synthesized (see Fig. 6).

When the cells were preincubated with CO rather than with H_2 as electron donor the ATP level and the membrane potential were already high before start of the reaction with CH_3I. As we have recently shown the oxidation of CO to CO_2 in acetate grown cells of *M. barkeri* is coupled with the generation of an electrochemical proton potential ($\Delta\tilde{\mu}_H+$) which drives the phosphorylation of ADP [18].

Inhibition of acetate synthesis by propyl iodide and reactivation by photolysis

Propyl iodide (100 μM) specifically inactivated acetate grown cells of *M. barkeri* with respect to their ability to mediate methanogenesis from acetate [43] and the CO_2/acetate exchange reaction ([12], Table II). (Methanogenesis from CO_2 and from methanol was not affected by the alkyl halide.) The rates of inactivation of the two activities differed, however, significantly. At low propyl iodide concentrations (< 10 μM) methanogenesis from acetate was com-

Table II: Effect of propyl iodide on acetate formation from CH_3I (2 mM), CO_2, and H_2, on the isotopic exchange between CO_2 and C1 of acetate (50 mM), and on methane formation from acetate (50 mM) by cells of acetate grown *M. barkeri*. The assays were performed in sealed 25 ml serum bottles containing 4 ml cell suspension (1 mg protein per ml). The gas phase was 80% N_2 and 20% CO_2 at 140 kPa pressure and contained 0.6% H_2 when acetate formation from CH_3I, CO_2, and H_2 was to be studied.

Addition	Acetate formation from CH_3I, CO_2, and H_2	Isotopic exchange between CO_2 and C1 of acetate (nmol after 10 min)	CH_4 formation from acetate
none	1120	3040	3100
5 μM propyl iodide	720	3040	960
10 μM propyl iodide	560	2020	390
50 μM proypl iodide	240	1520	80
100 μM propyl iodide	200	540	20
200 μM propyl iodide	120	390	0

pletely blocked within few minutes, whereas the CO_2/acetate exchange reaction was only affected after 30 min. It has been shown that inhibition of methanogenesis from acetate and of the CO_2/acetate exchange reaction by propyl iodide can be abolished by short exposure of the cells to light [12].

The effect of propyl iodide on acetate synthesis from CH_3I, CO_2, and H_2 was studied. It was found that this reaction was inhibited by propyl iodide and that the inactivation kinetics were similar to those observed for the CO_2/acetate exchange reaction (Table II).

The activity mediating acetate formation from CH_3I, CO_2, and H_2 was completely restored when cells inactivated by propyl iodide (100 μM) were resuspended in propyl iodide free suspension buffer and subsequently illuminated for 60 s (at 0 °C or 37 °C) with light from two 150 W tungsten lamps (Fig. 3).

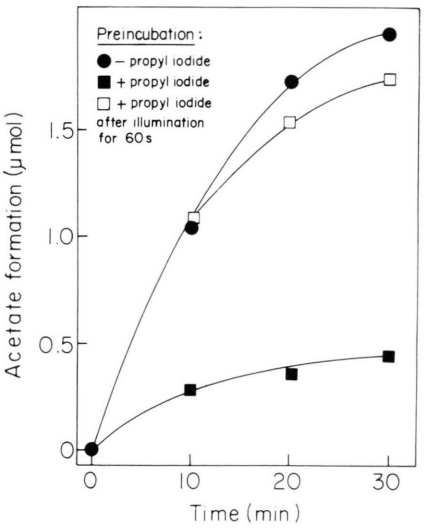

Fig. 3. Effect of illumination on acetate formation from CH_3I, CO_2, and H_2 by propyl iodide inactivated cells of acetate grown *M. barkeri*. The cells were preincubated in the dark at pH 7 and 37 °C with 100 μM propyl iodide for 15 min, collected by centrifugation, and resuspended in propyl iodide free imidazole phosphate suspension buffer. The assays were performed in the dark in sealed 25 ml serum bottles containing 4 ml cell suspension (0.8 mg protein per ml). The gas phase was 80% N_2, 20% CO_2, and 0.6% H_2 at 120 kPa pressure. The CH_3I concentration was 2 mM. Where indicated the complete assay was illuminated for 60 s at 0 °C with light from two 150 W tungsten lamps before start of the reaction by increasing the temperature to 37 °C.

Conclusions: corrinoid enzymes that mediate methyl transfer reactions are known to be inactivated by propyl iodide and to be reactivated by photolysis [44–47]. The findings thus suggest (see also Discussion) that a corrinoid is involved in acetate formation from CH_3I, CO_2, and H_2. This corrinoid is probably also involved in the CO_2/acetate exchange reaction since the exchange activity was inactivated by propyl iodide at the same concentrations and with similar kinetics. Methanogenesis from acetate was inhibited by propyl iodide at much lower concentrations than required to inhibit the CO_2/acetate exchange reaction or acetate formation from CH_3I, CO_2, and H_2 (Table II). This activity was also restored upon illumination. From this findings we conclude that *M. barkeri* contains at least two corrinoid enzymes that react with propyl iodide. The one corrinoid, which is inhibited by propyl iodide at low concentrations, is involved in methanogenesis from acetate rather than in acetate formation from CH_3I, CO_2, and H_2 or in the CO_2/acetate exchange reaction. The other corrinoid, which reacts with propyl iodide only at high concentrations, participates in all three reactions.

Inhibition of acetate synthesis by cyanide

Cyanide (20 μM) has been shown to specifically inactivate acetate grown cells of *M. barkeri* with respect to their ability to mediate methanogenesis from acetate and the CO_2/acetate exchange reaction [17, 48]. (Methanogenesis from CO_2 and from methanol was not affected by cyanide.) The rates of inactivation of the two activities by cyanide were almost identical [17]. It was found that acetate synthesis from CH_3I, CO_2, and H_2 was also inhibited by cyanide. Addition of cyanide resulted in a gradual decrease of the acetate formation rate rather than in an immediate cessation (Fig. 4). The rate of inactivation increased with increasing cyanide concentrations. The inactivation kinetics with cyanide were similar to those observed for methanogenesis from acetate and for the CO_2/acetate exchange reaction.

Conclusions: the findings are interpreted to indicate that cyanide exerts its inhibitory effect on methanogenesis from acetate, on the CO_2/acetate exchange reaction, and on acetate synthesis from CH_3I, CO_2, and H_2 at the same site. This site is probably the carbon monoxide dehydrogenase, which is known to be inactivated by cyanide [13, 49, 50].

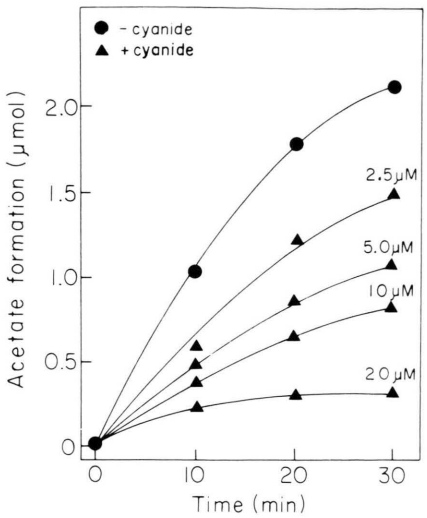

Fig. 4. Effect of cyanide on acetate formation from CH_3I, CO_2, and H_2 by cells of acetate grown *M. barkeri*. The assays were performed in sealed 25 ml serum bottles containing 4 ml of cell suspension (1 mg protein per ml). The gas phase was 80% N_2, 20% CO_2, and 0.6% H_2 at 120 kPa pressure. The CH_3I concentration was 2 mM. Cyanide was added directly before start of the experiments.

Inhibition of acetate synthesis by the protonophore TCS

The protonophore TCS was found to inhibit methanogenesis from acetate, the CO_2/acetate exchange reaction, and the formation of acetate from CH_3I, CO_2, and H_2 or from CH_3I, CO_2, and CO. Complete inhibition was observed at a TCS concentration of 1 nmol per mg protein of acetate grown cells. At this concentration $\Delta\Psi$ was found to be collapsed and the intracellular ATP level was very low (< 0.5 nmol ATP per mg protein).

The concentration of TCS required for half maximal inhibition of acetate formation from CH_3I, CO_2, and H_2 was significantly lower than for acetate formation from CH_3I, CO_2, and CO. This can be explained by the finding that CO oxidation to CO_2 is coupled with the generation of an electrochemical proton potential [18]. CO oxidation proceeded at a specific rate of 80–120 nmol·min^{-1}·mg protein^{-1}. Therefore, relatively more TCS should be required to collapse $\Delta\Psi$ in the presence of CO than in its absence.

Conclusions: these findings indicate that for the synthesis of acetate from CH_3I, CO_2, and reducing equivalents an electrochemical proton potential is required. The same holds true for methanogenesis from acetate and for the exchange reaction between CO_2 and the carboxyl group of acetate. It is of interest, in this respect, that methanogenesis from H_2 and methanol is not affected by TCS [41].

Inhibition of acetate synthesis by arsenate

Arsenate (K_i = 15 mM) was found to inhibit acetate formation from CH_3I, CO_2, and H_2 and from CH_3I, CO_2, and CO when the cells were incubated in the absence of phosphate. Inhibition of acetate synthesis was paralleled by a decrease of the ATP content and of $\Delta\Psi$.

Arsenate was shown to rapidly hydrolyze acetylphosphate and acetyl-CoA in cell extracts of acetate grown *M. barkeri* by the activity of phosphotransacetylase. The cells contained high specific activities of this enzyme (60–70 μmol·min^{-1}·mg protein^{-1}) and of acetate kinase (8–9 μmol·min^{-1}·mg protein^{-1}) [10].

Conclusions: it is concluded that arsenate exerts its inhibitory effect by hydrolyzing acetyl-CoA and acetyl-phosphate and by thus lowering the ATP level and $\Delta\Psi$. From the experiments with TCS it was concluded (see above) that acetate synthesis from CH_3I, CO_2, and reducing equivalents requires an electrochemical proton potential. The lowering of $\Delta\Psi$ by arsenate is therefore probably the reason why acetate synthesis was inhibited by arsenate.

Inhibition of acetate synthesis by the proton-translocating ATPase inhibitor DCCD

DCCD at a concentration of 100 nmol per mg cell protein was found to completely inhibit methanogenesis from acetate, the CO_2/acetate exchange reaction, and the formation of acetate from CH_3I, CO_2, and H_2 or from CH_3I, CO_2 and CO. The concentration of DCCD required for half maximal inhibition of acetate formation from CH_3I, CO_2, and H_2 was significantly lower than for acetate formation from CH_3I, CO_2, and CO (Fig. 5). This was paralleled by the finding that in the presence of H_2 as electron donor the membrane potential was collapsed by DCCD (30 nmol per mg protein), whereas, in the presence of CO the membrane potential remained at values near 110 mV (inside negative), due to the fact that the oxidation of CO to CO_2 is directly coupled with the generation of $\Delta\Psi$ [18].

For the interpretation of the following results it is important to know that ATP synthesis coupled to the oxidation of CO is driven by $\Delta\Psi$ *via* the membrane-bound ATP synthase [18] which is inhibited by DCCD [51]. When acetate grown cells of *M. barkeri* were incubated with CO (6%) and CO_2 (19%) in the presence of DCCD (30 nmol per mg protein, see Fig. 5), the intracellular ATP level decreased (Fig. 6). This shows that at the DCCD concentration used the ATP synthase was inhibited. Upon addition of CH_3I and onset of acetate formation the ATP level rapidly increased. In the first few minutes an apparent stoichiometry of 0.1 mol ATP per mol acetate synthesized was observed (Fig. 6) ($\Delta\Psi$ remained essentially constant).

Conclusions: it is concluded that ATP was generated *via* substrate-level phosphorylation during acetate formation from CH_3I, CO_2, and CO, since ATP was formed despite of the fact that the ATP synthase was inhibited by DCCD.

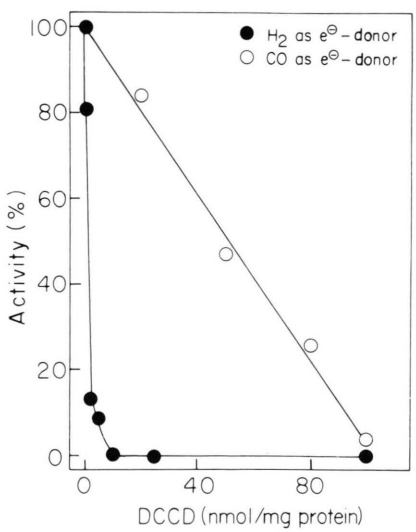

Fig. 5. Effect of DCCD on acetate formation from CH_3I, CO_2, and H_2 (●) and on acetate formation from CH_3I, CO_2, and CO (○) by cells of acetate grown *M. barkeri*. The assays were performed in sealed 120 ml serum bottles containing 4 ml of cell suspension (1 mg protein per ml). The gas phase was (●) 80% N_2, 20% CO_2, and 0.6% H_2 or (○) 75% N_2, 19% CO_2, and 6% CO at 120 kPa pressure. The cells were preincubated with DCCD for 10 min at 37 °C. Then the reaction was started by addition of CH_3I (2 mM). The amount of acetate formed was determined after 10 min. 100% activity = 30–32 nmol · min^{-1} · mg $protein^{-1}$.

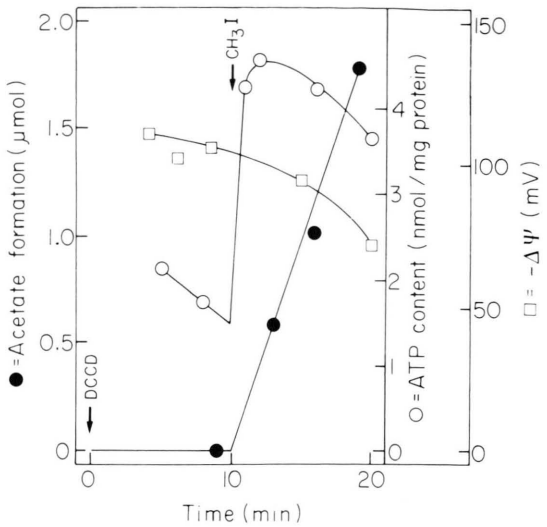

Fig. 6. Effect of DCCD on acetate formation from CH_3I, CO_2, and CO, on the cellular ATP content, and on $\Delta\Psi$. The assays were performed in sealed 120 ml serum bottles containing 6 ml of cell suspension of acetate grown *M. barkeri* (1 mg protein per ml). The gas phase was 75% N_2, 19% CO_2, and 6% CO at 120 kPa pressure. The cells were preincubated with DCCD (30 nmol per mg protein, added as ethanolic solution) for 10 min at 37 °C. Then the reaction was started with CH_3I (2 mM). The ATP content and $\Delta\Psi$ were determined in separate experiments.

Effect of arsenate on the inhibition of acetate synthesis by DCCD

It is shown in Fig. 5 that at high DCCD concentrations (100 nmol per mg protein) the synthesis of acetate from CH_3I, CO_2, and CO was severely inhibited. This inhibition was much less pronounced in the presence of arsenate at a concentration (10 mM) which only slightly inhibited acetate formation in the absence of DCCD. In the presence of arsenate the ATP level was only 0.5 nmol per mg protein, whereas, in its absence it was 3.5 nmol per mg protein (Fig. 7).

Conclusions: we assume that arsenate exerted its stimulatory effect on acetate formation from CH_3I, CO_2, and CO in the presence of DCCD by lowering the cellular ATP content.

When H_2 rather than CO was used as electron donor different results were obtained. DCCD at a concentration of 30 nmol per mg protein completely inhibited acetate formation from CH_3I, CO_2, and H_2 (Fig. 5). Under these conditions the ATP content of the cells was below 0.5 nmol per mg protein and $\Delta\Psi$ was below 50 mV (inside negative). Arsenate did not relieve this inhibition by DCCD.

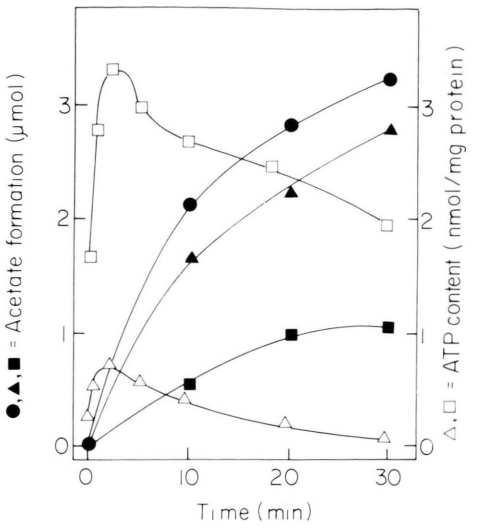

Fig. 7. Effect of arsenate on acetate formation from CH_3I, CO_2, and CO by DCCD inactivated cells of *M. barkeri*. The assays were performed in sealed 120 ml serum bottles containing 5 ml of cell suspension (0.8 mg protein per ml) in a 45 mM potassium morpholinopropanesulfonate buffer pH 7.4 (see Methods section). The gas phase was 75% N_2, 19% CO_2, and 6% CO at 120 kPa pressure. The final pH was 7.0. Where indicated the cells were preincubated with potassium arsenate (10 mM) and/or DCCD (100 nmol per mg protein, added as ethanolic solution) for 10 min at 37 °C before start of the reaction with CH_3I (2 mM).
(●) Acetate formation in a control;
(■) acetate formation in the presence of DCCD;
(▲) acetate formation in the presence of DCCD and arsenate;
(□) ATP content in the presence of DCCD;
(△) ATP content in the presence of DCCD and arsenate.

Discussion

First the mechanism of acetate formation from methyl iodide, CO_2, and reducing equivalents is discussed. Then the results are interpreted with respect to the mechanism of methanogenesis from acetate.

Acetate formation from CH_3I, CO_2, and reducing equivalents

Acetate grown cells of *Methanosarcina barkeri* mediated the formation of acetate from CH_3I (methyl group), CO_2 (carboxyl group), and reducing equivalents in a reaction coupled with the synthesis of ATP. The reaction was inhibited (or enzymes involved inactivated) by propyl iodide, by cyanide, by the protonophore TCS, by arsenate, and by the proton-translocating ATPase inhibitor DCCD. Propyl iodide inactivation was abolished upon illumination, suggesting that a corrinoid enzyme is the site of propyl iodide inhibition. Acetate synthesis from CH_3I, CO_2, and H_2 was more sensitive (lower K_i values) to TCS and DCCD than acetate formation from CH_3I, CO_2, and CO (*e.g.* Fig. 5). Inhibition by DCCD was partially relieved in the presence of arsenate, when CO rather than H_2 was the electron donor.

The experiments with DCCD indicated that ATP formation coupled to the synthesis of acetate did not involve the proton-translocating ATPase. The experiments with DCCD and with arsenate suggested that ATP was formed *via* substrate-level phosphorylation involving phosphotransacetylase and acetate kinase. The experiments with TCS and DCCD in the absence and presence of CO showed that an electrochemical proton potential ($\Delta\tilde{\mu}_H+$) was required for acetate synthesis from CH_3I, CO_2, and reducing equivalents. Free CO and free formate were excluded as intermediates in acetate synthesis.

These results are consistent with the pathway of acetate synthesis from CH_3I, CO_2, and H_2 as depicted in Fig. 8.

We propose that CH_3I reacts with a corrinoid enzyme X to yield CH_3X (reaction (i)). This enzyme also reacts with propyl iodide and is then inhibited. CO_2 is reduced to CO–Y (CO in a bound form) *via* carbon monoxide dehydrogenase (Y), in a reaction driven by the electrochemical proton potential ($\Delta\tilde{\mu}_H+$). Therefore, cyanide (*via* inactivation of carbon monoxide dehydrogenase) and TCS (*via* dissipation of $\Delta\tilde{\mu}_H+$) inhibited acetate synthesis. CH_3X and CO–Y react with CoA to give acetyl-CoA which, *via* acetyl-phosphate, is converted to acetate, yielding ATP *via* substrate-level phosphorylation. In the presence of arsenate the phosphotransacetylase catalyzed the hydrolysis of acetyl-CoA to acetate [10], therefore in the presence of arsenate no ATP can be generated (Fig. 8). The ATP formed in the acetate kinase reaction is proposed to be hydrolyzed *via* the proton-translocating ATPase thus generating the electrochemical proton potential ($\Delta\tilde{\mu}_H+$) required for the reduction of CO_2 to the level of bound CO (CO–Y) (Fig. 8). This explains why DCCD inhibited acetate formation from CH_3I, CO_2, and H_2.

When CO rather than H_2 was the electron donor $\Delta\tilde{\mu}_H+$ was additionally generated during CO oxidation to CO_2 [18]. This explains our finding that in the presence of CO higher concentrations of TCS and DCCD were required to inhibit acetate formation.

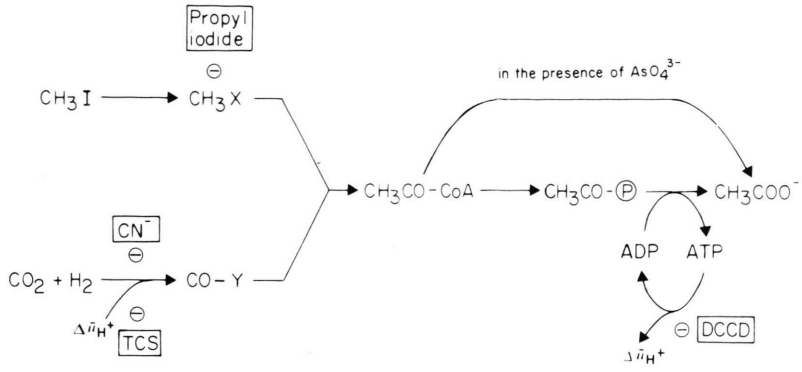

Fig. 8. Proposed pathway of acetate formation from CH_3I, CO_2, and H_2 in acetate grown *Methanosarcina barkeri*. Inhibition experiments with propyl iodide indicate that X is a corrinoid; Y is probably carbon monoxide dehydrogenase.

Inhibition of acetate formation from CH_3I, CO_2, and CO by DCCD at high concentrations was probably the result of the accumulation of ATP as indicated by the observation that arsenate was able to relieve this inhibition (Fig. 7).

Methanogenesis from acetate and the CO_2/acetate exchange reaction

Acetate formation from CH_3I, CO_2, and reducing equivalents, methanogenesis from acetate, and the CO_2/acetate exchange reaction share many properties in common. The three reactions were only catalyzed by acetate grown cells of *M. barkeri* rather than by cells grown on other methanogenic substrates. The three reactions were inhibited (or enzymes involved inactivated) by propyl iodide (activities being restored upon illumination), by cyanide, by TCS, by arsenate, and by DCCD, whereas, *e.g.* methanogenesis from CH_3OH and H_2 is not or only slightly (DCCD) [28, 41] affected by these inhibitors. The three reactions involve a bound C_1 unit probably at the oxidation level of CO (CO–Y) rather than free CO or free formate [52–54] as intermediate.

These findings indicate that the three reactions are catalyzed by common enzymes. Therefore, the results obtained for all three reactions can be interpreted with respect to the mechanism of methanogenesis from acetate. In Fig. 9 a pathway of methanogenesis from acetate accounting for all the data is shown.

The results indicate that in *M. barkeri* acetate is activated *via* acetyl-phosphate to acetyl-CoA at the expense of 1 mol ATP before being cleaved to CH_3X and CO–Y (reactions (b–d)). X is most probably the corrinoid enzyme that reacts with propyl iodide at high concentrations. The methyl group is transferred to CoM *via* a second corrinoid enzyme ([Co]E), which reacts with propyl iodide at lower concentrations. Methyl CoM is then reduced to methane in a reaction coupled with the generation of an electrochemical proton potential ($\Delta\tilde{\mu}_H+$) [28, 41]. The reducing equivalents required for this reaction are provided by CO–Y [55], which is oxidized to CO_2 in a reaction also generating $\Delta\tilde{\mu}_H+$ [18]. The electrochemical proton potential in turn drives the phosphorylation of ADP.

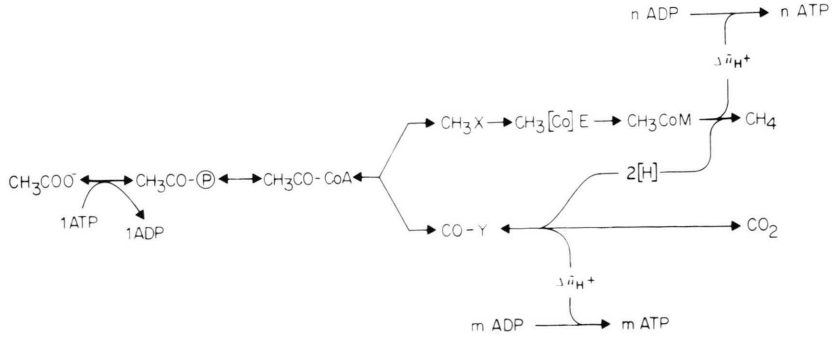

Fig. 9. Proposed pathway of methanogenesis from acetate in acetate grown cells of *Methanosarcina barkeri*. Inhibition studies with propyl iodide indicate that X is a corrinoid different from the corrinoid enzyme [Co] E; Y is probably carbon monoxide dehydrogenase. For ATP stoichiometries see the text: $1.5 > m + n > 1$.

The free energy change associated with methanogenesis from acetate in *M. barkeri* (reaction (a); $\Delta G'_o = -36$ kJ/mol) allows the net synthesis of 0.3–0.5 mol ATP per mol acetate [56]. Since per mol of acetyl-CoA formed from acetate 1 ATP is consumed in the acetate kinase reaction it is concluded that the $\Delta\tilde{\mu}_H+$ generated in the CH_3CoM reductase reaction and in the carbon monoxide dehydrogenase reaction must be sufficient to drive together the synthesis of 1.3–1.5 ATP per acetate. It has been shown that the acetoclastic methanogen *Methanothrix soehngenii* contains high activities of acetate thiokinase rather than phosphotransacetylase [57]. Assuming that in this organism acetate is activated by acetate thiokinase it must be postulated that the CH_3CoM reductase reaction and the carbon monoxide dehydrogenase reaction must be sufficient to drive together the synthesis of 2.3–2.5 ATP per acetate. Stoichiometries with fractional numbers are possible in this chemiosmotic mechanism of ATP synthesis [56, 58].

Acetate grown cells of *M. barkeri* were found to contain high specific activities of acetate kinase (8–9 μmol · min^{-1} · mg protein^{-1}) and phosphotransacetylase (60–70 μmol · min^{-1} · mg protein^{-1}) [10]. Both enzymes catalyze reversible reactions [58]. (The acetate kinase in addition mediates an exchange between acetate and acetyl-phosphate [59]). The free energy change ($\Delta G'_o$) associated with acetyl-CoA formation from acetate, CoA, and ATP *via* acetate kinase and phosphotransacetylase is 4 kJ/mol [58]. The interconversion of acetate and acetyl-CoA in acetate grown *M. barkeri* is therefore considered to proceed reversibly. The reaction leading to the formation of CH_3X and CO–Y from acetyl-CoA and the reaction leading to the formation of CO_2 from CO–Y must also be reversible since we consider them to be involved in acetate synthesis, acetate cleavage, and the CO_2/acetate exchange reaction.

Acknowledgements

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