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Methane formation from acetyl phosphate in cell extracts of *Methanosarcina barkeri*

Dependence of the reaction on coenzyme A

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Cell extracts (100000 × *g* supernatant) of acetate grown *Methanosarcina barkeri* (strain MS) catalyzed the formation of methane from acetyl phosphate (apparent $K_m \sim 2$ mM) with specific activities up to 50 nmol·min^{-1}·mg protein^{-1}, which is 25% of the specific rate of methane formation from acetate by intact cells. Methane formation from acetyl phosphate was strictly dependent on coenzyme A (CoA) (apparent $K_m \sim 0.1$ mM), which was demonstrated with extracts pretreated with charcoal to remove endogenous CoA. The extracts contained high specific activities of acetate kinase (16 μmol·min^{-1}·mg protein^{-1}) and of phosphotransacetylase (26 μmol·min^{-1}·mg protein^{-1}). The findings indicate that methanogenesis from acetate proceeds via acetyl phosphate and acetyl-CoA as intermediates. Almost identical results were obtained with cell extracts of acetate grown *M. barkeri* (strain Fusaro).

Acetyl-CoA; Acetyl phosphate; Coenzyme A; Methanogenesis; Coenzyme M; (*Methanosarcina barkeri*)

1. INTRODUCTION

Methanosarcina and *Methanothrix* spp. ferment acetate to CH_4 and CO_2, the methyl group being converted to CH_4 and the carboxyl group to CO_2 [1].

$$CH_3COO^- + H^+ \longrightarrow CH_4 + CO_2$$

$$\Delta G^{\circ\prime} = -36 \text{ kJ/mol}$$

Methyl-coenzyme M has been shown to be an intermediate in this metabolic pathway [2,3], indicating that acetate is cleaved such that the methyl group primarily yields a C_1 unit at the oxidation level of methanol and that therefore the carboxyl group must yield a C_1 unit at the oxidation level of CO (= formate). In a redox process the two C_1 units are then converted to CH_4 and CO_2, respectively [4].

Acetoclastic methanogens contain high amounts of the nickel-protein carbon monoxide dehydrogenase [5–7]. The enzyme is induced (5-fold) upon growth of the bacteria on acetate [8]. Antibodies against this enzyme inhibit methane formation from acetate in cell extracts [9]. These findings indicate that the nickel protein is involved in methanogenesis from acetate.

The carbon monoxide dehydrogenase from methanogens is similar to that found in *Clostridium thermoaceticum*, where it catalyzes the reversible formation of acetyl-CoA from CoA, CO and a methyl group attached to a corrinoid protein [10,11]. It has been proposed, but not yet demonstrated, that carbon monoxide dehydrogenase in acetoclastic methanogens mediates the same reaction and that, therefore, acetyl-CoA rather than free acetate should be the substrate of

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the C-C-cleavage reaction in methanogenesis from acetate [2,12]. Consistent with acetyl-CoA being an intermediate is the finding that *Methanosarcina* spp. contain high specific activities of acetate kinase and of phosphotransacetylase [13,14] and *Methanothrix* spp. high specific activities of acetyl-CoA synthetase [6].

Recently Krzycki and Zeikus [9,15] reported that cell extracts of *M. barkeri* (strain MS) catalyzed methanogenesis from acetate only when ATP was present. Specific activities of 20 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$ were reached. Acetyl phosphate could substitute for acetate plus ATP. Methane formation from acetate and from acetyl phosphate was not stimulated by the addition of CoA. These and other findings [4] clearly indicate that acetate has to be activated before it can be converted to CH_4 and CO_2. It remained to be shown, however, whether acetyl phosphate or acetyl-CoA is the substrate of the C-C-cleavage reaction.

In the above experiments with cell extracts, high concentrations of protein (20–30 mg/ml) were employed. The extracts were not dialyzed or otherwise freed from low molecular mass compounds. It thus has to be considered that the extracts contained sufficient CoA to saturate the reaction. We have therefore repeated these experiments with CoA free extracts and have found that methanogenesis from acetyl phosphate is strictly dependent on CoA.

During preparation of this manuscript a report has appeared that in cell extracts of *M. barkeri* methanogenesis from acetate is stimulated by CoA [3]. The specific activity of methane formation in these experiments was, however, only in the order of 1 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$ and stimulation by CoA was maximally 2.5-fold making the interpretation of these results ambiguous.

2. MATERIALS AND METHODS

3,3′,4′,5-Tetrachlorosalicylanilide (TCS) was from Eastman Kodak Co. (Rochester, USA). Monensin, activated charcoal (untreated powder) and coenzyme M (2-mercaptoethanesulfonate, sodium salt) were from Sigma (Deisenhofen, FRG). ATP (disodium salt), acetyl phosphate (K^+, Li^+) and CoA (free acid) were from Boehringer (Mannheim, FRG). *Methanosarcina barkeri* (strain MS) (DSM 800) [16] and *M. barkeri* (strain Fusaro) (DSM 804) [17] were from the Deutsche Sammlung von Mikroorganismen (Göttingen, FRG). The bacteria were grown on acetate at 37°C as described [18]. A 5% inoculum was used. The protein content of the bacteria and of the extracts was determined with Bio-Rad dye reagent (Bio-Rad Laboratories, München, FRG) using ovalbumins as standard [4].

2.1. *Experiments with cell suspensions*

For the experiments with cell suspensions strain MS was grown for 7–8 days to a cell concentration of approx. 80 mg protein/l and strain Fusaro for 4–5 days to a cell concentration of 100 mg protein/l. The cells were harvested by centrifugation at 3000 × *g* for 20 min and then resuspended in imidazole phosphate buffer (20 mM imidazole, 20 mM NaH_2PO_4, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 40 mM NaCl, 4 mM dithiothreitol and 15 μM resazurin, adjusted to pH 7 with NaOH) to a final concentration of 1–2 mg protein/ml and maintained under N_2 (gas phase) at 4°C for no longer than 1 h before use.

Methane formation from acetate in cell suspensions was assayed in 120 ml serum bottles containing 4 ml assay mixture: 30 mM sodium acetate, 20 mM imidazole, 20 mM NaH_2PO_4, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 40 mM NaCl, 4 mM dithiothreitol, 15 μM resazurin, adjusted to pH 7 with NaOH, and cell suspension, 1–2 mg protein. Except where otherwise indicated, the gas phase was N_2 at 120 kPa. The reaction was started by addition of the cell suspension and by increase of the temperature from 4 to 37°C. During incubation the serum bottles were shaken at 200 rpm. In 5 min intervals 0.3 ml gas samples were withdrawn and analyzed for CH_4 by gas chromatography [19].

2.2. *Experiments with cell extracts*

For the experiments with cell extracts strain MS was grown for 11–14 days to a cell concentration of 120 mg protein/l and strain Fusaro for 8–9 days to a concentration of 150 mg protein/l. The cells were harvested by centrifugation at 3000 × *g* for 20 min, washed once in 50 mM Mops buffer (50 mM morpholinopropane sulfonic acid, 10 mM $MgCl_2$, 4 mM dithiothreitol, 15 μM resazurin, adjust to pH 7 with KOH) and then resuspended in the same buffer to a final concentration of approx. 30 mg protein/ml. The suspension was then passed twice through a French pressure cell at 1400 × 10^5 Pa. Cell debris were removed by centrifugation at 27000 × *g* for 30 min. The supernatant is referred to as cell extract. Where indicated the cell extract was centrifuged at 100000 × *g* for 1 h (= 100000 × *g* supernatant) or treated with charcoal: 10 mg/ml extract; incubated for 30 min at 4°C and then removed by centrifugation at 27000 × *g* for 30 min (= charcoal treated cell extract). All preparation steps were performed under strictly anaerobic conditions at 4°C with H_2 as gas phase.

Methane formation from acetyl phosphate in cell extracts was assayed in 8 ml serum bottles containing 220 μl assay mixture: 30 mM acetyl phosphate, 0.3 mM CoA, 1 mM CoM, and 200 μl cell extract (or 100000 × *g* supernatant or charcoal-treated extract). Where indicated the concentrations of acetyl phosphate and of CoA were varied. The gas phase was H_2 at 200 kPa. The reaction was started by addition of the cell extracts and by increase of the temperature from 4 to 37°C. During incubation the vials were shaken at 700 rpm. At the times indicated 0.3 ml gas samples were withdrawn and analyzed for CH_4 by gas chromatography [19]. In successive determinations a correction was made for the amount of CH_4 already withdrawn.

3. RESULTS

In the following experiments it was essential that all preparation steps and the assays were performed under strictly anaerobic conditions. The serum bottles, the rubber stoppers, the syringes and all solutions used were maintained at least 2 days in an anaerobic chamber and dithiothreitol was added only after anaerobic conditions had already been achieved. If shortcuts from this procedure were taken the specific activities of methane formation were significantly lower. This was especially true for the experiments with cell extracts, where the activity decreased to < 1 nmol·min^{-1}·mg protein^{-1} when any of the precautions to exclude molecular oxygen were neglected.

3.1. *Experiments with cell suspensions*

Cell suspensions of *M. barkeri* (strain MS) and of *M. barkeri* (strain Fusaro) mediated the formation of methane from acetate with almost the same specific activity of approx. 200 nmol·min^{-1}·mg protein^{-1}. Methanogenesis from acetate was completely inhibited by the protonophore TCS (0.5 nmol/mg protein) and by the sodium ionophore monensin (25 nmol/mg protein). The two strains differed with respect to their sensitivity towards H_2. Methanogenesis from acetate by the Fusaro strain was completely blocked when 1% H_2 was present in the gas phase [20]. At this H_2 concentration the MS strain was practically unaffected. Only acetate grown cells mediated methane formation from acetate. H_2/CO_2 grown bacteria were inactive.

3.2. *Experiments with cell extracts*

Cell extracts of the two strains of acetate grown *M. barkeri* catalyzed the formation of methane from acetyl phosphate (fig.1A). The pH optimum was between 6.6 and 6.8. The specific activity increased with increasing protein concentrations to become constant above 25 mg/ml. The maximal specific activity reached was 50 nmol·min^{-1}·mg protein^{-1}, which is 25% of the specific rate of methane formation from acetate in cell suspensions of these organisms. The 100000 × *g* supernatant catalyzed the reaction at the same specific rates.

From Lineweaver-Burk plots an apparent K_m for acetyl phosphate of 2–3 mM was determined (fig.1B). Acetate (30 mM) plus ATP (15 mM) could substitute for acetyl phosphate. In the absence of ATP, however, acetate was not converted to methane. Extracts from H_2/CO_2 grown bacteria neither mediated methane formation from acetyl phosphate nor from acetate plus ATP.

The extracts of acetate grown bacteria contained high specific activity of acetate kinase (strain MS: 16 μmol·min^{-1}·mg protein^{-1}; strain Fusaro: 9 μmol·min^{-1}·mg protein^{-1}). The apparent K_m for acetate and for ATP were 7 and 2 mM, respectively (data for strain Fusaro). In H_2/CO_2 grown cells the specific activity was only 7% of that in acetate grown cells [21]. These findings indicate that acetate kinase is involved in methanogenesis from acetate.

In all the experiments with cell extracts reported until here the assay system was supplemented with CoA (0.3 mM). When this coenzyme was omitted, the specific activity was always lower, the stimulation by CoA being most pronounced at low protein concentrations. The latter finding indicated that the cell extracts contained endogenous CoA required for methanogenesis from acetyl phosphate. To test this assumption the extracts were pretreated with charcoal to adsorb the endogenous CoA. Such CoA free extracts catalyzed the formation of methane from acetyl phosphate only at very low rates but full activity was restored upon addition of CoA, stimulation was up to 20-fold. Half-maximal activity was obtained at CoA concentrations of approx. 0.1 mM (fig.2).

The cell extracts contained high specific activity of phosphotransacetylase (strain MS: 26 μmol·min^{-1}·mg protein^{-1}; strain Fusaro: 67 μmol·min^{-1}·mg protein^{-1}). The apparent K_m for acetyl phosphate and for CoA were 0.6 and 0.2 mM, respectively (data for strain Fusaro). In H_2/CO_2 grown cells the specific activity was only 30% of that in acetate grown cells [21]. These findings indicate that phosphotransacetylase has a function in methanogenesis from acetate.

Methanogenesis was also observed with acetyl-CoA (2 mM) rather than acetyl phosphate plus CoA was added to the extracts. The specific activity was, however, only 5–10% of that observed with acetyl phosphate plus CoA. Acetyl-CoA in the extracts was rapidly converted to CoA which probably inhibited the reaction [10]. In the

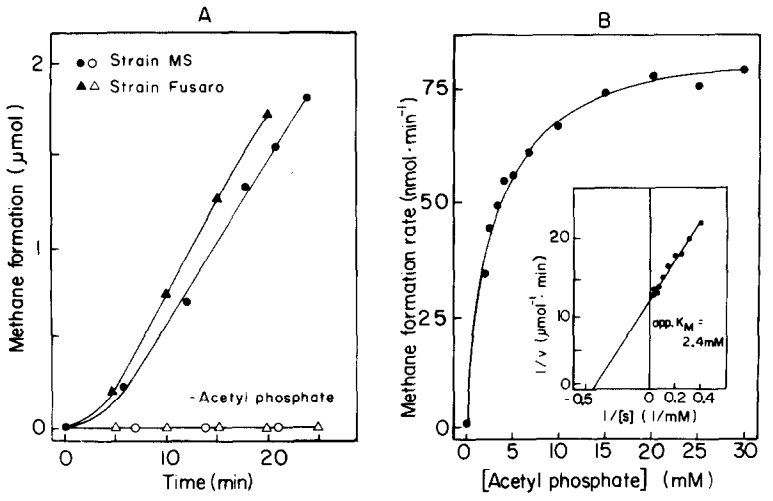

Fig.1. Methane formation from acetyl phosphate in cell extracts of *Methanosarcina barkeri* (strains MS and Fusaro). (A) Time course of the reaction. The assay mixture (220 μl) contained 12 mg protein/ml in the case of strain MS and 17 mg/ml in the case of strain Fusaro. [Acetyl phosphate] = 30 mM; [CoA] = 0.3 mM. (B) Dependence of the methane formation rate on the concentration of acetyl phosphate (data for strain MS). The inset shows a double reciprocal plot of the same data. The assay mixture (210 μl) contained 14 mg protein/ml. [Acetyl phosphate] = 0–30 mM; [CoA] = 0.3 mM.

presence of acetyl phosphate the free CoA concentration was kept low by the action of phosphotransacetylase.

Methanogenesis from acetyl phosphate in cell extracts of both strains was strictly dependent on the presence of H_2. Half-maximal rates were obtained with 25% H_2 in the gas phase. Another difference vs intact cells was that in cell extracts TCS (10 nmol/mg protein) and monensin (50 nmol/mg protein) did not inhibit methane formation.

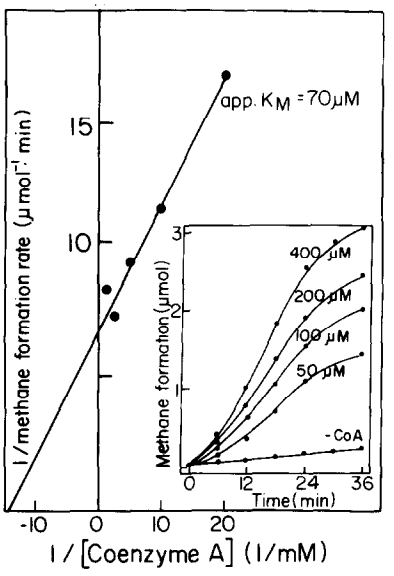

Fig.2. CoA dependence of methane formation from acetyl phosphate in charcoal-treated cell extracts of *M. barkeri* (strain MS). The inset shows the time course of the reaction. The assay mixture (220 μl) contained 19 mg protein/ml; [acetyl phosphate] = 30 mM, [CoA] = 0–0.4 mM.

4. DISCUSSION

The results of these and of previous investigations [3,4,9,15] with cell extracts of *M. barkeri* can be summarized as follows: (i) cell extracts incubated under strictly anaerobic conditions and with H_2 in the gas phase, can catalyze the formation of methane from acetate at specific rates similar to those in vivo; the membrane fraction (100000 × *g* sediment) is not required for activity; ionophores do not inhibit the reaction. (ii) Only extracts from acetate grown cells are active. When cells of *M. barkeri* are transferred from a methanol or H_2/CO_2 medium to an acetate medium the enzymes acetate kinase (14-fold), phosphotransacetylase (3-fold) and carbon monoxide dehydrogenase (5-fold) are 'induced'. (iii) Methane formation from acetate in cell extracts is strictly dependent on ATP and on CoA whereby acetate plus ATP can be substituted by acetyl phosphate. These results indicate that methanogenesis from acetate

in *M. barkeri* proceeds via acetyl phosphate and acetyl-CoA as intermediates and that acetyl-CoA is probably the activated species of acetate which is cleaved into the two C_1 units converted to CO_2 and CH_4. These findings support the proposal that carbon monoxide dehydrogenase in acetoclastic methanogens catalyzes the same reaction as the respective enzyme from *Clostridium thermoaceticum*.

Baresi [22] has reported that cell extracts of *M. barkeri* (strain 227) catalyzed methane formation from acetate (1–2 $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$) in the absence of ATP and that the membrane fraction was required for methanogenesis. H_2 inhibited the reaction. The latter finding indicates that the activity was probably derived from intact cells which were still present in his preparation.

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