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A methyl-CoM methylreductase system from methanogenic bacterium strain Göl not requiring ATP for activity

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Crude inside-out vesicles from the methanogenic strain Göl were prepared via protoplasts. These vesicles catalyzed methane formation from methyl-CoM and H_2 at a maximal rate of 35 nmol/min · mg protein. Methane formation by the vesicles did not depend on the addition of ATP. This was in contrast to conventionally prepared crude extracts from the same organism or from *Methanosarcina barkeri* which exhibited strict ATP dependence of methanogenesis. ATP analogues inhibited methanogenesis by extracts to a much higher extent than that by vesicles. Both, particulate and soluble components prepared from the crude vesicles by ultracentrifugation were necessary for ATP-independent methane formation from methyl-CoM and H_2. Hydrogenase activity was mainly associated with the particulate fraction whereas methyl-CoM methylreductase could be assigned to the soluble fraction. The detergent sulfobetaine inhibited methane formation from methyl-CoM without affecting hydrogenase or titanium citrate-dependent methylreductase activities, indicating that an additional membraneous component is involved in methanogenesis for methyl-CoM and H_2.

Methane formation; Methyl-CoM methylreductase; Hydrogenase; (Methanogenic bacteria)

1. INTRODUCTION

The methyl coenzyme M methylreductase system as first described [1] catalyzes the formation of methane from methyl coenzyme M with H_2 as electron donor:

$$CH_3\text{-S-CoM} + H_2 \longrightarrow CH_4 + \text{HS-CoM} \quad (1)$$

When measured in cell extracts of methanogenic bacteria this system exhibits a requirement for non-stoichiometric amounts of ATP [2] and is inhibited in its activity by ATP analogues [3].

The methylreductase system was identified as the site of ATP synthesis in methanogenic bacteria such as *Methanosarcina barkeri* or strain Göl and evidence for a chemiosmotic mechanism of ATP synthesis in methanogenic bacteria was obtained [4]. In this context the ATP requirement of the system in vitro is peculiar. It could result from some sort of disintegration of an enzyme complex during cell disruption. Results in favor of this assumption are presented in this paper because subcellular preparations of the methylreductase system can be made which do not require ATP for activity.

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Abbreviations: Na-CoM, sodium 2-mercaptoethane sulfonate; F_{420}, the (*N*-L-lactyl-γ-L-glutamyl)-L-glutamic acid phosphodiester of 7,8-didemethyl-8-hydroxy-5-deazariboflavin-5′-phosphate; methyl-CoM, methyl coenzyme M; β,γ-methylene-ATP, adenylyl-(β,γ-methylene)diphosphate; β,γ-imido-ATP, adenylyl-(β,γ-imido)-diphosphate; diadenosine-P_5, P^1,P^5-di-(adenosine-5′-)pentaphosphate; dial-ATP, adenosine 5′-triphosphate-2′,3′-dialdehyde; DTE, dithioerythritol; PMSF, phenylmethanesulfonyl fluoride; sulfobetaine, *N*-tetradecyl-*N*,*N*-dimethylammonio-3-propanesulfonate; HS-HTP, 7-mercaptoheptanoylthreonine phosphate

2. MATERIALS AND METHODS

2.1. *Strains and growth of organisms*

M. barkeri strain Fusaro (DSM 804) was obtained from the German Culture Collection (DSM), Braunschweig, FRG. The methanogenic strain Göl was isolated by F. Widdel from the

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sewage plant in Göttingen, FRG. *M. barkeri* and strain Göl were grown on the media described previously, supplemented with 1 g/l of sodium acetate [5]. *M. barkeri* was grown in a 300-l fermentor. The cells were spun down and the cell pellet was directly transferred into liquid nitrogen and then stored at −70°C until used. Mass culturing of strain Göl was done in 20-l carboys. The cells were collected by centrifugation and directly used for protoplast formation.

2.2. *Preparation of cell suspensions, protoplasts, vesicles and crude extracts*

Cell suspensions of *M. barkeri* were prepared by thawing frozen cells at room temperature in an anaerobic buffer containing the minerals of the above medium and 2 ml titanium citrate solution [4]. The cells of strain Göl and *M. barkeri* were washed once with 40 mM K-phosphate buffer, pH 7, containing 20 mM $MgSO_4$, 0.5 M sucrose, 4.9 mM Na-CoM, 10 mM DTE, 0.1 mg/l resazurin. Cell extracts were prepared by adding a few crystals of DNase and passing the cells through a French pressure cell at 130 MPa. Cell debris and unbroken cells were removed by centrifugation. Protoplasts of strain Göl were prepared as described previously [6]. Vesicles were prepared following the method of [7]: after the addition of a few crystals of DNase and of PMSF in a final concentration of 0.5 μg/mg protein, the protoplasts were passed through a French pressure cell at 65 MPa. In order to remove protoplasts and cell debris the preparation was centrifuged at 27000 × *g*. The resulting cell-free supernatant is referred to as crude vesicles.

2.3. *Fractionation of crude vesicles and of crude extracts*

Crude vesicles or crude extracts were further fractionated by anaerobic ultracentrifugation at 120000 × *g* for 3 h at 4°C using a Beckman 60Ti or 50Ti rotor in a Sorval OTD 75 ultracentrifuge. Crude vesicles formed two distinguishable phases: the supernatant was carefully removed and is referred to as vesicular cytoplasm. The sedimented material in the lower quarter of the centrifuge was diluted by filling up the tube with 40 mM K-phosphate buffer, pH 7. After a second ultracentrifugation at 120000 × *g* for 90 min at 4°C the supernatant was discarded. The remaining lower quarter is referred to as washed vesicles. Crude extracts were centrifuged under identical conditions, the supernatant is referred to as cytoplasm and the pellet in an appropriate volume of K-phosphate buffer as washed membranes.

2.4. *Methylreductase and hydrogenase assays*

Unless otherwise stated, 0.2 ml of vesicles or extracts were transferred into 2.3-ml stoppered glass tubes and incubated at 37°C. H_2-dependent and Ti-citrate-dependent methylreductase activities were assayed under H_2 and N_2, respectively, by following methane production gas chromatographically as described [4]. Unless otherwise stated, the reaction was started by the addition of 50 mM methyl-CoM. Methylviologen-dependent hydrogenase was assayed in 1.6-ml anaerobic cuvettes gassed with H_2 and filled with 1 ml anaerobic 100 mM Na-Tricine buffer, pH 8.0, containing 5 mM DTE, 3 mM cysteine, and 5 mM methylviologen. Hydrogen oxidation was measured by following the reduction of methylviologen at 604 nm (ϵ = 13.9 $mM^{-1} \cdot cm^{-1}$). F_{420}-dependent hydrogenase activity was assayed under the same conditions except that the 100 mM Na-Tricine was replaced by the above 40 mM K-phosphate buffer containing in addition 0.7 mM F_{420}. The activity was followed at 420 nm (ϵ = 34.5 $mM^{-1} \cdot cm^{-1}$).

3. RESULTS

3.1. *Methanogenesis by vesicle preparations*

The rates of methanogenesis from methyl-CoM and H_2 by vesicles and from methanol and H_2 by protoplasts or whole cells were determined. At a protein concentration of 30 mg/ml they corresponded to 52 (cells), 44 (protoplasts), and 35 (vesicles) nmol CH_4/min · mg protein, respectively. It is obvious that vesicles maintained 67% of their methanogenic activity in comparison to whole cells. The relatively low rate of 52 nmol CH_4/min · mg protein observed in whole cells was due to the presence of 0.5 M sucrose which always led to a decline of the methane formation rate. At a protein concentration of 1 mg/ml and in the absence of sucrose whole cells of strain Göl produced methane from methanol + H_2 at a rate of 300–400 nmol/min · mg protein.

3.2. *ATP-independence of methane formation by vesicle preparations*

Vesicle preparations converted methyl-CoM and H_2 to methane without the addition of external ATP (fig.1). In contrast, methanogenesis by conventionally prepared extracts was clearly ATP-dependent as evidenced by a low rate of methane formation when no ATP was added or when an ATP trap consisting of hexokinase and glucose was present. This ATP trap was without effect in the vesicle preparation. The ATP trap reduced the ATP content to 1.2 μM. However, the apparent K_m of methylreductase activation by ATP in cell extracts was determined to be 1 mM. The amount found can, therefore, not be sufficient for methylreductase activation.

The effect of various ATP analogues, some of which have been shown to inhibit component A3 of the methylreductase system from *Methanobacterium thermoautotrophicum* [3], were tested. It is evident from table 1 that the maximal inhibition of methanogenesis observed was 20% in vesicles but 80% in extracts compared to controls. Dialdehyde-ATP did not inhibit methane formation by vesicles at all whereas extracts were inhibited by 62.4%.

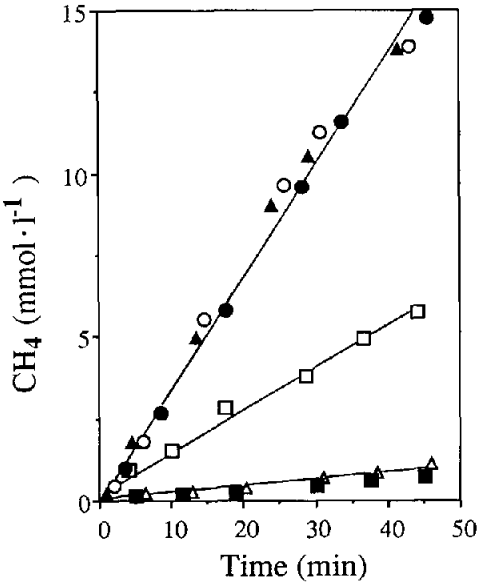

Fig.1. Effect of an ATP trap on methanogenesis from methyl-CoM + H_2 by crude vesicles or by crude extracts. Crude vesicles (protein concentration: 11.8 mg/ml) or crude extracts (protein concentration: 10.8 mg/ml) were suspended in a final volume of 0.5 ml K-phosphate buffer, pH 7, and preincubated at 37°C under H_2 either with 4 mM of ATP or with 14 U hexokinase and 10 mM glucose (ATP trap). The reaction was started by the addition of 50 mM methyl-CoM (t = 0 min). Crude vesicles: (▲) without additions, (●) with 4 mM ATP, (○) with ATP trap. Crude extracts: (△) without additions, (□) with 4 mM ATP, (■) with ATP trap.

3.3. *Fractionation of the vesicle preparation*

In order to locate the component responsible for the ATP independence, crude vesicles were fractionated by ultracentrifugation into soluble and particulate components. The same was done with cell extracts from *M. barkeri*.

Methanogenesis from methyl-CoM and H_2 by vesicular cytoplasm or washed vesicles alone proceeded at a low rate (table 2). However, the combination of the two fractions displayed ATP-independent methane formation. The methylreductase system of *M. barkeri* was entirely located in the cytoplasmatic fraction and was ATP-dependent. Most interestingly, the washed Göl vesicles could be substituted for by washed *M. barkeri* membranes without a loss of the ATP independence. In contrast, the combination of the cytoplasmic fraction from *M. barkeri* with Göl vesicles displayed ATP-dependent methanogenesis. This clearly demonstrates that the vesicular cytoplasm is responsible for the ATP independence of the system, but that membranes either from strain Göl or from *M. barkeri* are required for methane formation from H_2 + methyl-CoM.

Table 1

Effect of ATP analogues on methane formation by crude vesicles or crude extracts of strain Göl

ATP analogue (4 mM)	Methane formation rate			
	Crude vesicles		Crude extract	
	nmol/min · mg protein	%	nmol/min · mg protein	%
–	31.5	100.0	21.0	100.0
Dial-ATP	31.5	100.0	7.9	37.6
Diadenosine-P_5	30.4	96.5	7.8	37.1
β,γ-Methylene-ATP	28.5	90.4	6.9	32.9
β,γ-Imido-ATP	25.1	80.0	4.2	20.0

Crude vesicles (protein concentration: 27 mg/ml) or crude extracts (protein concentration: 22.5 mg/ml) were preincubated for 15 min with 2 mM ATP and 4 mM of the ATP analogue given under H_2 in a final volume of 0.2 ml. The reactions were started by the addition of 50 mM methyl-CoM

3.4. *The role of membraneous components in the methylreductase reaction with H_2 as electron donor*

It is apparent from table 2 that most of the hydrogenase activity of the complete methylreductase system of strain Göl was contributed by washed vesicles. The question was then whether additional components of the washed vesicles were required. Therefore the detergent sulfobetaine was tested as to its effect on the H_2-dependent methylreductase system and the methylreductase, assayed with titanium citrate as electron donor as well as on the activity of the hydrogenase. The H_2-dependent methylreductase system consisting of washed vesicles and vesicular cytoplasm lost 90% of its activity when the detergent was added to a final concentration of 0.5 mg/mg membrane protein (table 3). In contrast, the methylreductase activity with titanium citrate as electron donor and the methylviologen-dependent or the F_{420}-dependent hydrogenase activities remained largely unaffected. The combination of sulfobetaine-treated or untreated washed membranes from *M. barkeri* with Göl vesicular cytoplasm led to analogous results.

Table 2

Hydrogenase and H_2-dependent methylreductase activities by cytoplasmatic fractions and membrane fractions from *M. barkeri* or strain Göl or combinations thereof[a]

Source of fraction		Additions	μmol/min · assay	
Cytoplasm	Membranes or washed vesicles		Hydrogenase	Methylreductase
Göl[b]	–	ATP	0.5	5.2×10^{-3}
Göl[b]	–	ATP trap	0.5	5.8×10^{-3}
–	Göl[c]	ATP	2.3	$<1.0 \times 10^{-3}$
–	Göl[c]	ATP trap	2.3	$<1.0 \times 10^{-3}$
Göl[b]	Göl[c]	ATP	2.8	54.2×10^{-3}
Göl[b]	Göl[c]	ATP trap	2.8	45.5×10^{-3}
M. barkeri[d]	–	ATP	3.9	26.3×10^{-3}
M. barkeri[d]	–	ATP trap	3.9	1.2×10^{-3}
–	*M. barkeri*[e]	ATP	2.3	$<1.0 \times 10^{-3}$
–	*M. barkeri*[e]	ATP trap	2.3	$<1.0 \times 10^{-3}$
M. barkeri[d]	*M. barkeri*[e]	ATP	6.2	37.8×10^{-3}
M. barkeri[d]	*M. barkeri*[e]	ATP trap	6.2	2.9×10^{-3}
Göl[b]	*M. barkeri*[e]	ATP	2.8	67.2×10^{-3}
Göl[b]	*M. barkeri*[e]	ATP trap	2.8	68.8×10^{-3}
M. barkeri[d]	Göl[c]	ATP	6.2	33.0×10^{-3}
M. barkeri[d]	Göl[c]	ATP trap	6.2	2.3×10^{-3}

[a] All combined and uncombined fractions given in the table were suspended in a final volume of 0.2 ml. After incubating the preparations for 15 min at 37°C under H_2, the reactions were started by addition of 50 mM methyl-CoM to assay methylreductase activity. MV-dependent activity was tested as described in section 2. Where indicated an ATP trap consisting of 14 U hexokinase and 10 mM glucose or 2.5 mM ATP was added

[b] The protein concentration in the preparation was 3.5 mg/0.2 ml

[c] Washed vesicles; protein concentration was 0.5 mg/0.2 ml

[d] The protein concentration in the preparation was 3.5 mg/0.2 ml

[e] Washed membranes; protein concentration was 0.5 mg/0.2 ml

Table 3

Effect of sulfobetaine on hydrogenase and on H_2- or titanium citrate-dependent methylreductase activities

Sulfobetaine	Titanium citrate	Hydrogenase activity[a]		Methylreductase activity (nmol/min · mg protein)
		MV	F_{420}	
–	–	14.8	0.8	24.1
+	–	16.7	1.0	2.5
+	+	16.7	1.0	16.2

[a] Results expressed as μmol/min · ml

Methylreductase activity was determined by measuring the methane produced from methyl-CoM by vesicular cytoplasm (3 mg of protein) combined with washed vesicles (1 mg of protein) in a final volume of 0.2 ml with either H_2 or titanium citrate as electron donor. The reaction mixtures were preincubated under H_2. Where indicated washed vesicles were pretreated with 0.5 mg of sulfobetaine per mg protein. The reaction was started by the addition of 50 mM methyl-CoM

4. DISCUSSION

Two in-vitro systems leading to methane formation from methyl-CoM have been described. The H_2-dependent methylreductase system as present in cell extracts of *Mb. thermoautotrophicum* and other methanogens consists of several protein components, requires ATP and catalyzes methane formation in two steps (reactions 2 and 3) [8]:

components A_1, A_2, A_3 and C, ATP

$$CH_3\text{-S-CoM} + \text{HTP-SH} \longrightarrow CH_4 + \text{HTP-S-S-CoM} \quad (2)$$

$$\text{HTP-S-S-CoM} + H_2 \longrightarrow \text{CoM-SH} + \text{HTP-SH} \quad (3)$$

A system taking advantage of vitamin B_{12a} and

DTT or of titanium citrate as electron donor requires only component C and is ATP-independent [9] (reaction 4 and 5):

$$CH_3\text{-S-CoM} + \text{HTP-SH} \xrightarrow{\text{component C}} CH_4 + \text{HTP-S-S-CoM} \quad (4)$$

$$\text{HTP-S-S-CoM} + DTT_{red} \xrightarrow{B_{12a}} \text{CoM-SH} + \text{HTP-SH} + DTT_{ox} \quad (5)$$

In this publication a third system is described which works with H_2 as electron donor but is ATP-independent. It is prepared by the gentle disruption of protoplasts of the methanogenic bacterium strain Göl. More drastic treatments of strain Göl also lead to ATP-dependent preparations. The results show that the ATP-dependence must be connected with a component which is not tightly membrane-bound. This follows from the observation that membrane fragments or washed vesicles stimulated ATP-independent methanogenesis when added to vesicular cytoplasm of strain Göl. Therefore, it is the preparation of the cytoplasmic fraction via protoplasts and vesicles that leads to the ATP-independent methylreductase system. The recently described enzyme of *Mb. thermoautotrophicum* which reduces the heterodisulfide HTP-S-S-CoM with H_2 is not stimulated by ATP [10]. So ATP might be involved in multienzyme complex formation. Such complexes have been detected in strain Göl and designated as methanoreductosome [11]. Their reorganization in conventionally prepared extracts of methanogens might require ATP.

The H_2-dependent methylreductase system described here exhibits a requirement for an as yet unidentified membraneous component. Hydrogenase plus methylreductase activities are not sufficient for H_2-dependent formation of methane from methyl-CoM: a component which is inactivated by the detergent sulfobetaine is required in addition. This finding makes a further study of the vesicular H_2-dependent methylreductase system in connection with its role in energy conservation very attractive.

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