Methane synthesis by membrane vesicles and a cytoplasmic cofactor isolated from *Methanobacterium thermoautotrophicum*

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Methanobacterium thermoautotrophicum when grown on ordinary culture medium has a tough cell wall which is lysozyme-resistant and difficult to disrupt by physical means. The cell wall, however, can be weakened by the addition of D-sorbitol to the growth medium and the organisms form protoplasts after lysozyme addition. This technique allowed the isolation of two types of intracellular small vesicles: (a) isolated by disruption of the total cell population (lysozyme-sensitive and lysozyme-resistant cells) by ultrafrequency sound and (b) isolated by osmotic lysis of protoplasts. For the first time, a small vesicle fraction isolated as in (a) was capable of synthesizing methane from CO₂ and H₂ without cytoplasm. There was, however, an absolute requirement for a small, heat-stable, oxygen-sensitive cofactor which was isolated from the cytoplasm. Methane synthesis with this vesicle fraction was inhibited by the detergent deoxycholate, and by the protonophores 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone. Mg²⁺-ATPase appeared to be located on the outer or cytoplasmic surface of the small vesicle fraction isolated as in (b). The results were consistent with a previously made suggestion [Sauer, Erfle & Mahadevan (1981) *J. Biol. Chem.* **256**, 9843–9848] that the interior of the small intracellular vesicles becomes acid during methane synthesis.

Methanobacterium thermoautotrophicum when grown on standard medium has a tough cell wall which requires drastic procedures for complete disruption. This has hindered the isolation of internal membrane structures which are required for the biosynthesis of methane from CO₂. As a result the methane-synthesizing system has not been well characterized and to date the reduction of CO₂ to methane requires crude cell extracts (Leigh & Wolfe, 1983).

This paper describes a new technique for weakening the cell wall of *M. thermoautotrophicum*. This facilitates the isolation of intracellular vesicles which are competent to synthesize methane and appear to have right-side-out orientation. For methane synthesis these vesicles required a new, heat-stable, oxygen-sensitive cofactor which was isolated from the cytoplasm. These results support previous reports (Doddema *et al.*, 1979, 1981; Sauer *et al.*, 1980*a,b*, 1981) that in this organism, hydrogen oxidation, energy transduction and methane synthesis are associated with intracellular membrane structures.

Experimental

Materials

RNAase, DNAase, bovine pancreas lysozyme (mucoprotein *N*-acetylmuramoyl-hydrolase, EC 3.2.1.17) (grade 1 lyophilized) and carbonyl cyanide *m*-chlorophenylhydrazone were obtained from Sigma. 2,4-Dinitrophenol was obtained from ICN Pharmaceuticals, Montreal, Que., Canada. Dicyclohexylcarbodi-imide was from Eastman Organic Chemicals, Rochester, NY, U.S.A. Percoll, a colloidal silica coated with poly(vinylpyrrolidone) was from Pharmacia (Canada), Dorval, Que., Canada. Titanium citrate (0.1 M) was prepared from TiCl₃ and citrate (Zehnder & Wuhrmann, 1977) and adjusted to pH 7.0.

Bacterial culture

M. thermoautotrophicum (A.T.C.C. 29183) cell lines were started from single colonies. Agar plates (3%, w/v) were prepared with the standard medium of Zeikus & Wolfe (1972) and stored in an anaerobic chamber (Coy Laboratory Products,

Ann Arbor, MI, U.S.A.) in an atmosphere of N_2/H_2 (9:1). When oxygen-free, the plates were streaked with the organism and while still in the hood, placed in a Parr 920 ml cell disruption bomb and sealed. The bomb was thoroughly flushed with CO_2/H_2 (1:4), pressurized to 3×10^3 kPa, sealed and incubated at $65^\circ C$ until colonies appeared on the agar plates. Single colonies were transferred to liquid medium in the anaerobic hood. Growing cells were then serially transferred as described (Sauer *et al.*, 1980a) and used to inoculate large fermenters.

The standard medium of Zeikus & Wolfe (1972) was used in the large (14 litre) fermenters as described (Sauer *et al.*, 1980) except that nickel ($1 \mu M$) was added (Diekert *et al.*, 1981). For larger batch preparations the cells were grown in a 75-litre fermenter (New Brunswick Scientific, Edison, NJ, U.S.A.).

When cells were grown for protoplast and vesicle isolation, 50 mM-D-sorbitol was added to the standard medium (Zeikus & Wolfe, 1972) in the large (14 or 75 litre) fermenters. The cells were harvested, after 36–48 h, in a continuous-flow high-speed centrifuge (CEPA model Z-41) (New Brunswick Scientific) under a flow of N_2 . At this stage, cells grown in sorbitol-containing medium formed protoplasts (60–70%) when lysozyme was added. If harvesting of these cells was delayed, after 48 h the cells began to form protoplasts spontaneously and there was a significant loss in methane production.

Cells were routinely stored in 100 ml sealed flasks at $0-2^\circ C$ under CO_2/H_2 (1:4). As the cells slowly oxidized H_2 even at this temperature, the gas phase had to be replenished at weekly intervals.

Protoplasts and intact cells were counted by phase-contrast microscopy in a standard counting chamber with a Thoma grid.

Vesicle isolation

For the preparation of vesicles only cells grown in the sorbitol medium were used. The sorbitol medium produced a mixture of *M. thermoautotrophicum* cells; some formed protoplasts when lysozyme was added while the remainder were lysozyme-resistant. All the cells grown in sorbitol medium had a fragile cell wall and therefore were easily disrupted.

Protoplasts and lysozyme-susceptible cells generally had little methane-synthesizing capacity. Not surprisingly, vesicles isolated from such cells were relatively inactive in making methane. In contrast, lysozyme-resistant, sorbitol-grown cells, which also have a fragile cell wall, yielded vesicles which had good methane-producing activity. Accordingly, two methods were used for vesicle isolation. In the first, the total cell population was

disrupted by sonication and vesicles were obtained which had the capacity to synthesize methane. In the second, cells were converted to protoplasts with lysozyme then lysed by osmotic shock. Intracellular vesicles were then obtained from the lysate. With this technique, lysozyme-resistant cells were discarded.

Method 1. Stored cells (60 ml, 12 mg of protein/ml) were centrifuged at $26000g$ for 15 min and resuspended in 50 ml of CO_2 - and O_2 -free buffer (0.1 M-KCl/0.1 M-potassium phosphate, pH 7.0) reduced with titanium citrate (0.2 mM). The buffers were kept fully reduced, by using Resazurin as indicator. The cells were disrupted by ultra-frequency sound as described (Sauer *et al.*, 1979) with total sonication time limited to 3 min. Unbroken cells and cell debris were removed by centrifugation at $36000g$ for 20 min and the supernatant fraction (the $36000g$ cell extract) was carefully transferred into 13 ml polyallomer tubes and sealed in the anaerobic hood. The pellet was discarded. The tubes were centrifuged in a fixed-angle titanium rotor at $378000g$ for 3 h in a Beckman L2-75-B preparative ultracentrifuge. The supernatant fraction ($378000g$ supernatant) was removed and the tightly packed pellet was resuspended in a small volume of the KCl/phosphate buffer and stored under H_2 at $2^\circ C$. This fraction is referred to as 'sonicate vesicles'. All operations were done in the anaerobic hood.

Method 2. Cells from the fermenters were sedimented by centrifugation at $26000g$ for 15 min and resuspended in 400 ml of 0.1 M-potassium phosphate (pH 7.0)/0.4 M-D-sorbitol buffer at a cell concentration of approx. 1.5 mg of protein/ml. The temperature of the cell suspension was raised to $45^\circ C$ and lysozyme ($50 \mu g/ml$), DNAase ($10 \mu g/ml$), and RNAase ($10 \mu g/ml$), were added. After 30 min, when approx. 65% of the cells had formed protoplasts, the suspension was centrifuged at $105000g$ for 30 min. The pellet was resuspended in 140 ml of distilled water in order to lyse the protoplasts. The suspension was then centrifuged at $12000g$ in order to precipitate any unbroken cells and protoplast remnants. Protoplast remnants were readily identified by phase-contrast microscopy as large membrane 'ghosts', the cytoplasmic membrane fraction. This $12000g$ pellet was sealed anaerobically in 38 ml Beckman polyallomer tubes and suspended in 40% (v/v) Percoll/50 mM-Tris, pH 7.0, containing 50 mM-sorbitol. Self-generating gradients were formed by centrifugation at $85000g$ for 45 min. The cytoplasmic membrane fraction separated in a well-defined band. The $12000g$ supernatant fraction or lysate, which contained the small vesicles, was centrifuged at $378000g$ for 3 h. The resulting pellet (lysate vesicles) was stored as above. The $378000g$

supernatant fraction was used for hydrogenase assays.

Electron-microscope studies

The sectioning and staining of all fractions for electron-microscopic examination were done as described by Sauer *et al.* (1977). For the preparation of negatively stained grids, cells and protoplasts were suspended in 2% potassium phosphotungstate solution, placed on a carbon-coated grid and allowed to settle. Excess solution was removed and the grids were allowed to air dry.

Isolation of cytoplasmic cofactor

For the preparation of cytoplasmic cofactor, *M. thermoautotrophicum* cells were resuspended in 25 mM-potassium phosphate buffer (pH 7.0) fully reduced with titanium citrate and broken in a Ribi cell fractionator (model R7-1; Norwalk, CT, U.S.A.) exactly as described (Sauer *et al.*, 1980a). Subsequent operations were done in the anaerobic hood. The broken cell suspension was adjusted to pH 1.5 with 6 M-HCl, centrifuged to remove cell debris and denatured protein and adjusted to pH 7.0. For the preparative isolation of cytoplasmic cofactor, a DEAE-Sephadex column (20 cm $\times$ 2.5 cm) (DEAE-Sephadex A-25, Pharmacia) was equilibrated with 1 M-NaCl, then washed with 25 mM-potassium phosphate, pH 7.0, in 10 mM mercaptoethanol until the effluent was chloride-free. The sample was pumped on the column and eluted with a linear gradient of 25 mM-potassium phosphate buffer, pH 7.0 (500 ml) and 1 M-NaCl in 25 mM-potassium phosphate buffer, pH 7.0 (500 ml) reduced with 10 mM-mercaptoethanol. Fractions (12 ml) were collected in 1.8 cm $\times$ 15 cm aluminium seal culture tubes. The tubes were sealed, removed through the air-lock, and assayed for activity with sonicate vesicles. For analytical work the same procedure was followed except that the cytoplasmic cofactor was eluted from a smaller column (12 cm $\times$ 1.6 cm) with a linear gradient of 100 ml of 25 mM-potassium phosphate (pH 7.0) and 100 ml of 1 M-NaCl in 25 mM-potassium phosphate (pH 7.0) and titanium citrate (0.2 mM) was used as reductant. With the analytical column 6 ml fractions were collected.

For ultrafiltration, the 378000g supernatant fraction was transferred to a 65 ml Amicon stirred cell (model 52; Amicon Canada, Oakville, Ont., Canada) with the appropriate ultrafiltration membranes (YM10 or UM2) under a constant stream of N₂. The stirred cell and membrane were deoxygenated with titanium-reduced buffer before use. The filtrates were collected in stoppered bottles under a constant N₂ stream. Smaller volumes were filtered in a 12 ml stirred cell (model 12).

Assay procedures

Hydrogenase was measured by H₂ evolution. Sealed 5 ml incubation flasks (Sauer *et al.*, 1979) flushed with Ar contained 40 mM-Tris (pH 8.0)/15 mM-sodium dithionite/1 mM-Methyl Violögen in a final volume of 1.0 ml. The reaction was started by injecting enzyme protein (5–25 μ l). Incubations were with constant shaking at 60°C. H₂ was measured in a Fisher gas partitioner (Sauer *et al.*, 1979) at different time intervals to establish linear rates.

Mg²⁺-ATPase was assayed in 50 mM-Tris buffer (pH 8.0)/10 mM-MgCl₂/5 mM-ATP, with 0.25–0.50 ml of protein in a final volume of 1.0 ml. The enzyme was oxygen-stable and the reactions were carried out aerobically at 60°C for 10 and 20 min. The incubations were stopped with 1.0 ml of 10% (w/v) trichloroacetic acid and P_i was determined by the method of Fiske & Subbarow (1925). A blank with Mg²⁺ omitted was subtracted from every sample.

Methane production from isolated vesicles (sonicate or lysate vesicles) was measured in 1.0 ml Reacti-Vials (Pierce Chemical Co., Rochford, IL, U.S.A.) sealed with Teflon/silicone disks. Vials were flushed with O₂-free H₂ for a minimum of 3 min before use. Incubations were made in 0.1 M-KCl/0.1 M-potassium phosphate, pH 7.0, containing NaH¹⁴CO₃ (1 mM; 5 $\times$ 10⁵ d.p.m.), 2.5 mM-titanium citrate and either 0.2 ml of 378000g supernatant fraction or cytoplasmic cofactor in a final volume of 0.4 ml. If purified cytoplasmic cofactor was used, the incubation mixture contained 2.5 mM-ATP and 3 mM-MgCl₂ in addition to the above. ATP and Mg²⁺, although not required in incubations with newly isolated vesicles, occasionally stimulated methane synthesis in aged vesicle preparations. Incubations were in a shaking water bath for 20 min at 60°C. ¹⁴CH₄ analyses were done as described (Sauer *et al.*, 1977). Intact cells were incubated in 5 ml rubber-stoppered Erlenmeyer flasks as described previously (Sauer *et al.*, 1980a). Protein was measured by the method of Miller (1959).

Results

Properties of cells grown in D-sorbitol-containing medium

M. thermoautotrophicum grown in standard medium (Zeikus & Wolfe, 1972) is resistant to lysozyme (Doddema *et al.*, 1979). The cells however can be modified during growth by exposure to D-sorbitol so that protoplasts are formed (Figs. 1a and 1b). In a population of cells grown in D-sorbitol, methane production per cell

Fig. 1. *Electron-microscopic studies of M. thermoautotrophicum cells and protoplasts*

(a) Protoplasts of *M. thermoautotrophicum* shown by negative staining (bar indicates $0.42\ \mu\text{m}$). (b) *M. thermoautotrophicum* cells from a typical 14 litre fermentation flask after lysozyme addition. Shown in the section are a mixture of protoplasts and lysozyme-resistant cells (bar indicates $0.26\ \mu\text{m}$). (c) Upper band from a Percoll density gradient which was developed as described in Table 2 and which shows *M. thermoautotrophicum* protoplasts (bar indicates $0.26\ \mu\text{m}$). Lysozyme-resistant cells accumulate in the lower band of the Percoll gradient (results not shown).

was decreased when compared with cells grown in standard medium (Table 1). Lysozyme-resistant cells (25% of the population in the experiment in Table 1), however, retained good activity.

Table 2 shows the typical distribution of *M. thermoautotrophicum* cells and protoplasts on a 90% Percoll gradient. The upper and middle bands from the gradient contained the preponderance of protoplasts and some 'light' cells (sp. gr. 1.04–1.05) which were less active in synthesizing methane than the heavier lysozyme-resistant cells which were in the lower band (sp. gr. 1.09). Fig. 1(c) shows protoplasts recovered from the upper band of a Percoll gradient.

The low methane-producing capacity of protoplasts was reflected in the lysate vesicle fraction which was isolated from these protoplasts. Methane production from lysate vesicles was only 0.19 ± 0.03 ($n = 12$) nmol/10 min per mg of protein.

Table 1. *Methane synthesis by M. thermoautotrophicum cells grown with and without sorbitol*

Cells grown in sorbitol-containing medium were concentrated 10-fold into two sealed flasks. To the control flask was added RNAase and DNAase at 60°C . To the other flask was added RNAase, DNAase, and lysozyme at 60°C . Cells grown in standard medium were concentrated 10-fold. Cells were incubated in Tris (33 mM, pH 7.0), sorbitol (50 mM), $\text{NaH}^{14}\text{CO}_3$ (15 mM, 6×10^6 d.p.m.) in 5 ml sealed Erlenmeyer flasks under H_2 as described in the Experimental section.

Cells	$10^{-10} \times \text{Cells/ml}$	Methane synthesis (nmol/min per 10^{10} cells)
Cells grown with sorbitol		
Control	2.83	141
+ Lysozyme	0.72	555
Cells grown in standard medium	0.95	447

Table 2. *Distribution of M. thermoautotrophicum cells grown in sorbitol containing medium on a Percoll gradient* After addition of lysozyme, cells and protoplasts (31.3 mg of protein/tube) were suspended in 90% Percoll/0.2 M-sorbitol/0.1 M-potassium phosphate (pH 7.0) and anaerobically sealed in 30 ml Beckman polyallomer tubes. Self-generating gradients were formed by centrifugation at 105 000g for 30 min. Approximate buoyant densities were determined with marker beads. Gradient bands were removed and transferred in the anaerobic hood.

Distribution	Percoll gradient bands		
	Upper	Middle	Lower
Cells/ml ($\times 10^{-10}$)	2.0	9.0	2.1
Protoplasts/ml ($\times 10^{-10}$)	13.2	6.4	0.02
Protoplasts %	86.8	41.6	0.9
Approximate density	1.04	1.05	1.09
Methane synthesis (nmol/min per mg of protein)	16.0	54.6	506.0

Methane synthesis in sonicated cell extracts prepared by method 1

Sorbitol was required in the incubation medium in order to isolate active methane-producing vesicle preparations. As an example, cell extracts from *M. thermoautotrophicum* grown in the absence of sorbitol synthesized 0.55 nmol of methane/10 min per mg of protein (Sauer *et al.*, 1980a), whereas these same extracts prepared from cells grown in sorbitol medium synthesized 4.91 nmol of methane/10 min per mg of protein (Table 3).

Methane synthesis in the 36 000g cell extract was a function of the small vesicle fraction which sedimented at 378 000g. This fraction separated as a dark brown layer from the straw-coloured high-speed supernatant fraction. After 1 h centrifugation at 378 000g, methane-synthesizing activity in the original extract was decreased 62% as a result of sedimenting vesicles (Table 3). After 3 h centrifugation the vesicle fraction was completely sedimented as a tightly packed dark brown pellet which was devoid of methane-synthesizing activity, as was the 378 000g supernatant fraction. When the brown pellet, i.e. sonicate vesicles, was recombined with the 378 000g supernatant fraction, 53% of the original activity was restored. Some irreversible loss of activity resulted when the vesicle fraction was resuspended in KCl/phosphate buffer. Methane production by sonicate vesicles plus 378 000g supernatant fractions was 2.77 ± 0.22 ($n = 12$) nmol/10 min per mg of protein.

The sonicate vesicle fraction was unstable and retained the best activity when stored anaerobically at 2–4°C. Even so this fraction lost 50–75% of its activity within 1 week of isolation.

Effects of uncoupling agents on sonicate vesicles

Sonicate vesicles were responsive to uncoupling reagents (Table 4). The proton conductors 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone were powerful inhibitors

Table 3. *Resolution of methane synthesis in the 36 000g cell-free extract prepared from M. thermoautotrophicum by method 1*

The 36 000g supernatant fraction was obtained by centrifugation of bacterial cell extracts at 36 000g for 20 min. The preparation of 378 000g supernatant fraction, sonicate vesicles and details of the incubation procedure are described in the Experimental section.

Cell fraction	Methane synthesis (nmol/10 min per mg of protein)
36 000g cell extract	4.91
36 000g cell extract after centrifugation for 1 h at 378 000g	1.89
378 000g supernatant	<0.01
Sonicate vesicles (378 000g pellets)	<0.01
Sonicate vesicles (378 000g pellet) + 378 000g supernatant fraction	2.60

of methane synthesis (Table 4). Chloroform also inhibited methane synthesis, but its mode of action is not clear. Used alone, chloroform does not discharge a pH gradient, either in *M. thermoautotrophicum* cells (Sauer *et al.*, 1981) or in rat liver mitochondria (Rottenberg, 1983), but effectively decreases ATP concentration and energy charge (Sauer *et al.*, 1981).

Dicyclohexylcarbodi-imide specifically binds to a small polypeptide of the membrane F_0 component of the ATPase complex and has been shown to interfere with transmembrane proton conductance (Mao *et al.*, 1982). This reagent was not inhibitory with crude cell extracts (Sauer *et al.*, 1979) but was slightly inhibitory with sonicate vesicles (Table 4). As would be expected, the membrane-solubilizing detergent, deoxycholate, completely abolished methane synthesis with sonicate vesicles.

Table 4. *Effects of some inhibitors on methane synthesis by sonicate vesicles prepared by method 1*
Sonicate vesicles and 378000g supernatant fraction were incubated and assayed as described in the Experimental section.

Addition	Methane synthesis (nmol/10min per mg of protein)	Inhibition (%)
Expt. 1		
None	2.59	—
Dicyclohexylcarbodi-imide (40 μ M)	1.61	37.8
Carbonyl cyanide <i>m</i> -chlorophenylhydrazone (50 μ M)	0.42	83.4
Chloroform (50 μ M)	0.39	84.9
2,4-Dinitrophenol (100 μ M)	<0.01	100
Deoxycholate (0.25%)	<0.01	100
Expt. 2		
None	6.18	—
Carbonyl cyanide <i>m</i> -chlorophenylhydrazone (50 μ M)	1.03	83.3
2,4-Dinitrophenol (100 μ M)	<0.01	100

Table 5. *Distribution of hydrogenase and Mg²⁺-ATPase in subcellular fractions of M. thermoautotrophicum protoplasts*
Cell fractions were isolated and assayed as described in the Experimental section. Lysate vesicles were prepared by method 2 in the Experimental section. Fractions were checked for purity by phase-contrast microscopy. Where indicated, membrane fractions were sonicated for 1 min (30s on, 30s off) at 2/3 maximum power setting. A representative experiment is given from three similar experiments.

Cell fraction	Hydrogenase (μ mol/min per mg of protein)	Mg ²⁺ -ATPase (nmol/min per mg of protein)	
		Before sonication	After sonication
Lysate vesicles	2.82	12.1	12.5
Cytoplasmic membrane	0.97	0.3	2.8
High speed supernatant fraction	8.86	—	—

Hydrogenase and ATPase activities in lysate vesicles prepared by method 2

Vesicles isolated from bacterial cells which are disrupted by physical means, i.e. ultrafrequency sound or shear forces, generally assume both a right-side-out and inside-out orientation. Indeed a small vesicle fraction with Mg²⁺-ATPase activity has been isolated from *M. thermoautotrophicum* in which the vesicles had both right-side-out and inside-out orientation (Doddema *et al.*, 1978, 1979). Lysate vesicles (Table 5) which were isolated from *M. thermoautotrophicum* protoplasts were not subjected to drastic disruption procedures but instead were released from the protoplasts by osmotic lysis. The vesicle membrane therefore may well have the same orientation as in the intact cell. Certainly membrane vesicles prepared by osmotic lysis of *Escherichia coli* spheroplasts are predominantly in the right-side-out configuration (Kaback, 1971).

Hydrogenase activity as measured by H₂ evolution appeared to be loosely membrane-bound and significant quantities of this enzyme become soluble during isolation (Table 5) as has been suggested (Doddema *et al.*, 1979; McKellar & Sprott,

1979). Nevertheless, the results show that both hydrogenase and ATPase activities are components of the vesicle membrane and this suggests that these organelles, although inactive for methane synthesis, probably are fully competent to synthesize ATP.

In lysate vesicles, the Mg²⁺-ATPase apparently is located on the outer surface since the activity was not increased when the vesicles were completely disrupted by ultrafrequency sound. By contrast, the cytoplasmic membrane fraction was stimulated 9-fold by ultrasound treatment (Table 5) which suggests that the Mg²⁺-ATPase is located on the inner surface of the membrane. This placement of the Mg²⁺-ATPase on the two membranes is consistent with the possibility that lysate vesicles are invaginations of the cytoplasmic membrane and that these may separate and re-anneal during the isolation procedures.

Properties of cytoplasmic cofactor

Sonicate vesicles had an absolute requirement for 378000g supernatant fraction that was not replaced by ATP, Mg²⁺, FAD, 8-hydroxy-5-deazaflavin (*F*₄₂₀) or 2-mercaptoethanesulphonate

Table 6. *Methane synthesis by sonicate vesicles with either cofactors or 378000g supernatant fraction*

The incubation mixture contained sonicate vesicles, 0.1 M-KCl, 0.1 M-potassium phosphate buffer, pH 7.0 and cofactors, or 378000g supernatant fraction to a final volume of 0.4 ml. The incubation procedure is described in the Experimental section. Heated 378000g supernatant fraction was kept at 100°C for 5 min in a sealed tube under Ar. Aerobic 378000g supernatant fraction was exposed to air for 10 min until there was a pink Resazurin colour change. Acidified 378000g supernatant fraction was prepared as described in the Experimental section. A representative experiment is given from three similar experiments.

Additions	Methane synthesis (nmol/10 min per mg of protein)
None	<0.01
ATP (5 mM) + Mg ²⁺ (10 mM)	<0.01
FAD (10 mM) + F ₄₂₀ (2.5 mM)	<0.01
Coenzyme M (0.6 mM)	0.01
378000g supernatant (0.2 ml)	3.57
Heated 378000g supernatant (0.2 ml)	3.28
378000g supernatant, aerobic (0.2 ml)	0.43
Acidified 378000g supernatant (0.2 ml)	3.34

(coenzyme M) (Table 6). The active principle in the 378000g supernatant was heat- and acid-stable, provided that strictly anaerobic technique was used. The acid-treatment step removed protein without loss of activity. Even a brief exposure to air, however, resulted in an irreversible 88% loss of activity (Table 6). The active principle or cytoplasmic cofactor in the 378000g supernatant fraction was negatively charged and was eluted from an analytical column of DEAE-Sephadex with a linear NaCl gradient. All the active fraction was eluted as a single peak in 0.25 M-NaCl (fractions 8–12). On this column a blue fluorescent compound, a yellow non-fluorescent compound and a yellow fluorescent compound, all of which have been described (Keltjens *et al.*, 1983a,b) were eluted after the cytoplasmic cofactor. Cytoplasmic cofactor, as eluted from the column, was non-fluorescent. F₄₂₀ was eluted in fractions 25–28 and concentrated as described (Eirich *et al.*, 1978). Methane production from sonicate vesicles showed good linearity with cytoplasmic cofactor (Fig. 2).

The cytoplasmic cofactor was fairly stable when stored anaerobically at –20°C with approx. 75% of the activity remaining after 2 weeks. As isolated in its active form, the cytoplasmic cofactor was ninhydrin-negative and colourless, with absorption peaks at 296 nm and 253 nm. On oxidation, the absorption peaks shifted to 310 nm and 276 nm. With prolonged exposure to air the cytoplasmic cofactor turned yellow, then deep brown (F. D. Sauer, S. Mahadevan & J. D. Erfle, unpublished work).

Ultrafiltration experiments showed that the molecular mass of the cytoplasmic cofactor was between 1000 and 10000. All of the activity passed through an Amicon YM10 membrane which has a

Fig. 2. *Requirement for cofactor from 378000g supernatant for methane synthesis by sonicate vesicles prepared by method 1*

The active fractions eluted from DEAE-Sephadex were combined and concentrated 10-fold by passage through a UM2 Amicon ultrafiltration membrane. Activity was assayed as described in the Experimental section. In this experiment the vesicle fraction in the absence of cofactor synthesized 1.3 nmol of CH₄/h per mg of protein.

cut off of *M_r* 10000 when calibrated with globular solutes. When the YM10 filtrate which contained the cytoplasmic cofactor was subsequently filtered through a UM2 membrane (*M_r* 1000 cut off) all of the activity remained in the retentate. Passage through Amicon Diaflo Ultrafilters (UM2 or

YC05) was a practical means of concentrating the cytoplasmic cofactor in solution.

Discussion

M. thermoautotrophicum grown in standard media is resistant to lysozyme (mucopolysaccharide *N*-acetylmuramoylhydrolase, EC 3.2.1.17) (Doddema *et al.*, 1979). Lysozyme attacks murein-containing cell walls, i.e. cell walls with alternating residues of *N*-acetylglucosamine and *N*-acetylmuramic acid [2-amino-3-*O*-(1-carboxyethyl)-2-deoxy-D-glucose]. As recently reported (Hammes *et al.*, 1979; König & Kandler, 1979) the cell wall of *Methanobacterium* contains pseudomurein, which differs from murein in that D-amino acids are absent and that in the heteropolysaccharide strands *N*-acetylmuramic acid residues are replaced by *N*-acetylglucosaminuronic acid residues. They may account for the resistance of the unchanged *Methanobacterium* cell wall to lysozyme. As shown here, however, the *M. thermoautotrophicum* cell wall can be modified by exposure to D-sorbitol during growth in an as yet undetermined manner so that it is hydrolysed by lysozyme and protoplasts are formed.

ADP/ATP translocase, a mitochondrial enzyme which is rarely found in procaryotic cells, has been reported in *M. thermoautotrophicum* (Doddema *et al.*, 1981). This and other evidence led to the speculation that in *M. thermoautotrophicum* energy transduction and methane synthesis occur in intracellular organelles or vesicles (Sauer *et al.*, 1980a,b, 1981; Kell *et al.*, 1981).

The intracellular membrane vesicles of *M. thermoautotrophicum* contain all enzymes necessary to synthesize methane, including hydrogenase and have a Mg^{2+} -ATPase which appears to be located on the outer vesicle surface. From this one may postulate that H_2 oxidation proceeds through a membrane-spanning hydrogenase and results in an internal acidification of these vesicles, as has been proposed (Spencer *et al.*, 1980; Sauer *et al.*, 1981). ATP would then be synthesized by an outward movement of protons through the proton-translocating ATPase. Methane synthesis, if on the outer vesicle surface, would serve in the removal of translocated protons. It may be relevant that *E. coli* spheroplasts appear to have a membrane-spanning hydrogenase (Jones, 1980) and that when *E. coli* spheroplasts were pulsed with H_2 there was a rapid translocation of protons across the spheroplast membrane. The requirement for an ADP/ATP translocase in *M. thermoautotrophicum* however is not accounted for by this model, since ATP would be synthesized on the cytoplasmic side of the vesicle and there would be no requirement for a trans-membrane ADP/ATP interchange.

To date methane synthesis from CO_2 has only been possible with crude cell extracts which were either dialysed (Romesser & Wolfe, 1981) or passed through Sephadex G-25 (Leigh & Wolfe, 1983) in order to remove small cofactors. In the present report a method is presented for the preparation of a small vesicle fraction which can synthesize methane in the presence of a cytoplasmic cofactor but does not require cytoplasmic proteins.

The cytoplasmic cofactor is of low molecular mass, colourless, heat- and acid-stable, oxygen-sensitive and could not be replaced by Mg^{2+} , ATP, FAD, F_{420} , coenzyme M, or tetrahydrofolic acid (monoglutamate). It has a characteristic u.v. spectrum in both the reduced and oxidized state. The cytoplasmic cofactor differed from the known cofactors which stimulate methane synthesis and include CO_2 reduction factor, methanopterin (a 2-amino-4-hydroxypteridine) and methyl-coenzyme M (Leigh & Wolfe, 1983; Keltjens *et al.*, 1983a,b). Both methanopterin and CO_2 reduction factor were shown to be oxygen stable (Leigh & Wolfe, 1983) whereas the cytoplasmic cofactor is extremely oxygen sensitive. Methyl-coenzyme M can be ruled out by size since it would not be retained by the Amicon filters.

There was no indication that the sonicate vesicles were stimulated by any cofactor other than the cytoplasmic cofactor. Perhaps methanopterin, CO_2 reduction factor, and coenzyme M are solubilized and lost with the more drastic cell disruption methods, but can be retained by vesicles isolated with gentler disruption procedures from *M. thermoautotrophicum* cells grown in sorbitol.

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