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The role of tetrahydromethanopterin and cytoplasmic cofactor in methane synthesis

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A fraction previously isolated from acid-treated supernatant fraction of *Methanobacterium thermoautotrophicum* by DEAE-Sephadex chromatography [Sauer, Mahadevan & Erfle (1984) Biochem. J. **221**, 61–97] which was absolutely required for methane synthesis, has been separated into two compounds, tetrahydromethanopterin (H_4MPT) and an as-yet-unidentified cofactor we call 'cytoplasmic cofactor'. H_4MPT was identified by its u.v. spectrum and by ^{13}C- and ^1H-n.m.r. spectroscopy. The reduction of 2-(methylthio)ethanesulphonic acid (CH_3-S-CoM) to methane by the membrane fraction from *M. thermoautotrophicum* was completely dependent on the addition of cytoplasmic cofactor. Methane synthesis from CO_2, however, was only partially dependent on cofactor addition, and 57% of the original activity was retained in its absence. The kinetics of ^{14}C labelling were consistent with the scheme methyl-H_4MPT → CH_3-S-CoM → methane, as has been proposed. This is the first time that direct experimental evidence has been presented to show that the proposed methyl transfer from H_4MPT to coenzyme M (HS-CoM) actually occurs.

The structure of methanopterin (MPT), a new coenzyme required for methanogenesis, has recently been described (Keltjens *et al.*, 1983*a*,*b*; Van Beelen *et al.*, 1984*a*). The compound is biologically active only when reduced to tetrahydromethanopterin (H_4MPT) and undergoes distinctive u.v. spectral changes on oxidation (Escalante-Semerena *et al.*, 1984*a*; Sauer *et al.*, 1984).

We showed previously (Sauer *et al.*, 1984) that a cytoplasmic cofactor was required by the membrane fraction from *Methanobacterium thermoautotrophicum* in order for CO_2 reduction to methane to proceed. The isolation procedure, however, did not adequately separate the cofactor from H_4MPT, and the u.v. spectrum of H_4MPT was attributed to the cytoplasmic cofactor. We have now achieved the complete separation of H_4MPT from this cofactor and show that both are required for methane production.

EXPERIMENTAL

Isolation of cytoplasmic cofactor and H_4MPT

Acid-treated supernatant (ATS) fraction was prepared as described previously (Sauer *et al.*, 1984) from 60 g (wet wt.) of *M. thermoautotrophicum* cells. The ATS fraction was adjusted to pH 4.5 and pumped into a column (2.5 cm × 25 cm) of DEAE-Sephadex A-25 in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI, U.S.A.). The column was washed with 70 ml of water containing 10 mM-mercaptoethanol, followed by a linear gradient of 5 mM-potassium phosphate buffer, pH 6.5 (500 ml), and 0.6 M-NaCl in 5 mM-potassium phosphate buffer, pH 6.5 (500 ml), reduced with 10 mM-mercaptoethanol. The fractions (13 ml) were assayed for H_4MPT and cytoplasmic cofactor. Fractions containing the cofactor or H_4MPT were combined and desalted by repeated filtration in a 200 ml Amicon stirred cell (Amicon Canada, Oakville, Ont., Canada) with a YC05 ultrafiltration membrane (approx. M_r cut-off 500) to a NaCl concentration of 5 mM. The cofactor was adjusted to pH 6.5, and applied to a second DEAE-Sephadex A-25 column (1.5 cm × 25 cm) and eluted with a linear gradient of 5 mM-potassium phosphate buffer, pH 6.5 (100 ml) and 0.6 M-NaCl in 5 mM-potassium phosphate buffer, pH 6.5 (100 ml), reduced with 10 mM-mercaptoethanol. The fractions containing the cytoplasmic cofactor were combined and concentrated by ultrafiltration. All operations were performed in the anaerobic hood with exclusion of O_2 from all solutions to the greatest extent possible.

N.m.r. spectroscopy

^1H- and ^{13}C-n.m.r. spectra of H_4MPT were obtained on a Bruker WM 250 spectrometer operating at 250 and 62.8 MHz respectively. Chemical shifts are referenced to external TMS. Samples were prepared as described by Van Beelen *et al.* (1984*a*). After freeze-drying twice from ^2H_2O, approx. 10 mg was dissolved in 0.3 M-K_2HPO_4 solution in ^2H_2O, p^{2}H = 10.5, under anaerobic conditions and sealed in 5 mm-outer-diam. n.m.r. tubes. Coupling patterns in the ^1H-n.m.r. spectrum were confirmed by a homonuclear (^1H) correlation two-dimensional n.m.r. spectrum (COSY-45). The number of attached protons corresponding to each ^{13}C resonance was determined with the distortionless-enhancement-of-polarization-transfer pulse sequence (Doddrell *et al.*, 1982).

The M_r of H_4MPT was determined by f.a.b. m.s. on a Finnegan-MAT 410 mass spectrometer using a glycerol matrix.

Abbreviations used: H_4MPT, tetrahydromethanopterin; CH_3-S-CoM, 2-(methylthio)ethanesulphonic acid; HS-CoM, coenzyme M; ATS, acid-treated supernatant; TMS, tetramethylsilane; f.a.b. m.s., fast-atom-bombardment mass spectroscopy; CMC, critical micelle concentration.

Membrane isolation and enzyme assays

M. thermoautotrophicum cells were grown in sorbitol-containing medium (Sauer *et al.*, 1984) in a 75-litre fermenter and harvested after 48 h in a continuous-flow high-speed centrifuge (CEPA model Z-41; New Brunswick Scientific, Burlington, Ont., Canada), under a continuous flow of CO_2. The cells were disrupted and the membrane fraction isolated as described previously (Sauer *et al.*, 1984) under 'Method 1' for the preparation of 'sonicate vesicles'. Enzyme assays were done in 1.0 ml Reacti-Vials (Pierce Chemical Co., Rockford, IL, U.S.A.) at 60 °C with constant shaking in an atmosphere of O_2-free H_2. $^{14}CH_4$ analyses were done as described by Sauer *et al.* (1977).

Formation of methylene-H_4MPT and CH_3-S-CoM

Incubation mixtures that contained [^{14}C]methylene-H_4MPT and [^{14}C]CH_3-S-CoM were stopped with 1.6 ml of cold 100% methanol and centrifuged for 5 min in an Eppendorf Microfuge. The sample was flash-evaporated and injected with 0.25 ml of solvent in a Waters h.p.l.c. apparatus (model U6K injector, 6000 A solvent-delivery system and 450 variable wavelength detector; Waters Scientific Ltd., Mississauga, Ont., Canada) equipped with a reverse-phase octadecyl (5 μm) Hi-Chrom preparative column (25 cm × 1 cm internal diam.). The cofactors were eluted with O_2-free methanol (7.5%, v/v) in 25 mM-acetic acid, pH 4.5, at a flow rate of 1 ml/min. Samples were collected in sealed tubes which contained H_2. With this solvent system and flow rate, CH_3-S-CoM was eluted in 15.5 min, methylene-H_4MPT was eluted in 19.5 min, and H_4MPT was eluted in 43 min.

Samples that contained HS-CoM and H_4MPT derivatives were chromatographed on pre-coated silica-gel G plates (250 μm) in a solvent system of butan-1-ol/acetic acid/water (4:1:1, by vol.). Radioactive samples were identified by autoradiography and comparison with ^{14}C-labelled standards. CH_3-S-CoM had an R_F of 0.5 and methylene-H_4MPT had an R_F of 0.1 in this system. No attempt was made to separate methyl-H_4MPT from methylene-H_4MPT.

[^{14}C]CH_3-S-CoM isolated from reaction mixtures was also purified by high-voltage paper electrophoresis (Whatman 3 MM paper) (1000 V, 1 h) in 8% (v/v) formic acid together with authentic standards. In this system [^{14}C]CH_3-S-CoM migrated 10 cm toward the anode and was detected by autoradiography.

Materials

[^{14}C]Methylene-H_4MPT was prepared by allowing 4.74 μmol of H_4MPT in anoxic potassium phosphate buffer (0.1 M, pH 7.8) to react with 8.33 μmol of [^{14}C]formaldehyde (sp. radioactivity 10 mCi/mmol) under Ar gas. After completion of the reaction, the sample was diluted to 60 ml with 5 mM-potassium phosphate, pH 6.5, and applied to a DEAE-Sephadex column (2.5 cm × 20 cm) and washed with 50 ml of the same buffer. [^{14}C]Methylene-H_4MPT was eluted with a linear gradient of 500 ml of potassium phosphate (5 mM, pH 6.5) and 500 ml of 0.6 M-NaCl in potassium phosphate (5 mM, pH 6.5) reduced with 10 mM-mercaptoethanol. Fractions containing the labelled cofactor were concentrated and desalted by repeated ultrafiltration. [^{14}C]CH_3-S-CoM was prepared as described by Sauer *et al.* (1980). DEAE-Sephadex (Cl^- form) was from Pharmacia, Dorval, Que., Canada. The sulphobetaine zwitterionic surfactants (Gonenne & Ernst, 1978) were obtained from Calbiochem–Behring Corp., La Jolla, CA, U.S.A. [^{14}C]Formaldehyde (sp. radioactivity 40 mCi/mmol) and $Na_2{}^{14}CO_3$ (50 mCi/mmol) were purchased from the New England Nuclear, Lachine, Que., Canada.

RESULTS

The chemical structures of MPT (Van Beelen *et al.*, 1984*b*) and H_4MPT (Escalente-Semerena *et al.*, 1984*a*) have been described. H_4MPT has the u.v. spectrum initially described by us for the cytoplasmic cofactor (Sauer *et al.*, 1984) (Fig. 1). H_4MPT at pH 6.5 has absorption maxima at 301 nm, 249 nm and 219 nm and, when oxidized in air, the u.v. spectrum shifts to absorption maxima of 320 nm, 276 nm and 230 nm.

The ^{1}H-n.m.r. spectrum of H_4MPT shows the same essential features as those previously attributed to this compound (Escalante-Semerana *et al.*, 1984*a*). These features include the resonance at 1.24 p.p.m. attributable to the two methyl groups (positions 12 and 13 of the pteridine moiety), the proton at position 1 of the α-ribofuranose group at 5.10 p.p.m. as well as the pair of doublets at 6.6 and 7.1 p.p.m. (positions 3,5 and 2,6 respectively of the aniline moiety) and confirm the structure.

At 250 MHz the spectral resolution is insufficient to observe the protons at positions 6 and 7 of the pteridine ring that distinguish H_4MPT from MPT. However, the homonuclear correlation n.m.r. spectrum (COSY-45) showed similar coupling patterns to those observed for 5,10-methenyl-H_4MPT (Van Beelen *et al.*, 1984*b*) and, in addition, showed the presence of coupling between the methyl resonance at 1.24 p.p.m. and a multiplet at 3.5 p.p.m. in which the resonance of proton 7 of the

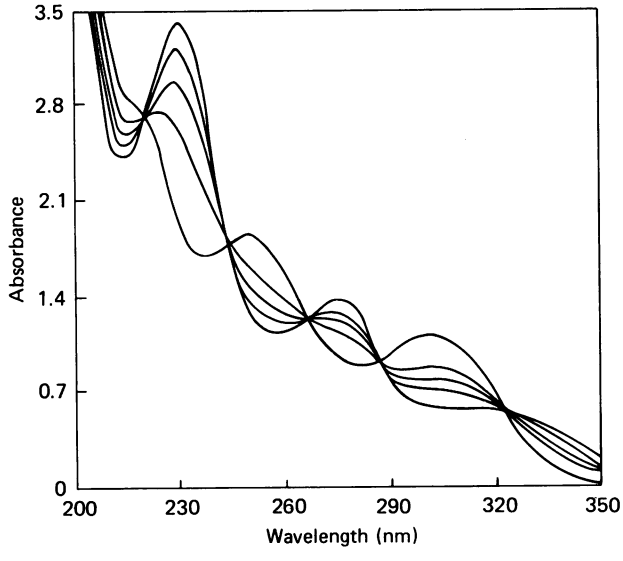

Fig. 1. Reduced and oxidized u.v. scan of H_4MPT

H_4MPT (50 μg) was scanned in a 1 cm cuvette in air at 4 min intervals in 5 mM-potassium phosphate buffer, pH 6.5. Absorption maxima for H_4MPT at 301, 249 and 219 nm. Absorption maxima of oxidized MPT at 320, 276 and 230 nm.

Table 1. 62.8 MHz ^{13}C chemical-shift assignments for H_4MPT*

Abbreviation used: n.d., not detected.

Carbon no.	^{13}C chemical shift (p.p.m. relative to TMS)	No. of attached protons
Pteridine moiety		
2	157.6	0
2	n.d.	
6	72.3	1
7	51.7	1
9	51.8	0
10	n.d.	
11	58.5	1
12	20.5	3
13	22.4	3
Aniline moiety		
1	144.3	0
2, 6	116.1	1
3, 5	130.6	1
4	141.8	0
Tetrahydroxypentane moiety		
1	37.2	2
2	70.5	1
3	74.5	1
4	73.2	1
5	69.4	2
α-Ribofuranose moiety		
1	102.4	1
2	71.3	1
3	69.9	1
4	84.0 (J_{PC} = 8.6 HZ)	1
5	65.3	2
α-Hydroxyglutarate moiety		
1	178.7	0
2	73.6 (J_{PC} = 5.2 HZ)	1
3	30.8	2
4	33.2	2
5	182.5	0

* Assignments based on those determined by Van Beelen *et al.* (1984*b*) for MPT and 5,10-methenyl-H_4MPT, which employed heteronuclear ($^1H/^{13}C$) two-dimensional n.m.r. spectra.

pteridine ring would appear (i.e. the 13,7 coupling of the pteridine ring). The additional coupling between the methyl at position 12 and the proton at position 11 of the pteridine ring was obscured by the H^2HO resonance. In the ^1H spectrum of H_4MPT, the two methyl resonances are coincident, whereas in the case of the 5,10-methenyl derivative they are resolved.

The assignment of the ^{13}C chemical shifts (Table 1) further confirms the structure as H_4MPT. Assignments were based on a comparison with those previously published for MPT and 5,10-methenyl-H_4MPT (Van Beelen *et al.*, 1984*b*). The determination of the number of attached protons corresponding to each carbon resonance confirms these assignments. Phosphorus–carbon couplings present on position 4 of the α-ribofuranose moiety and position 2 of the α-hydroxyglutarate group confirm the presence of a phosphate group. An f.a.b. mass spectrum of this

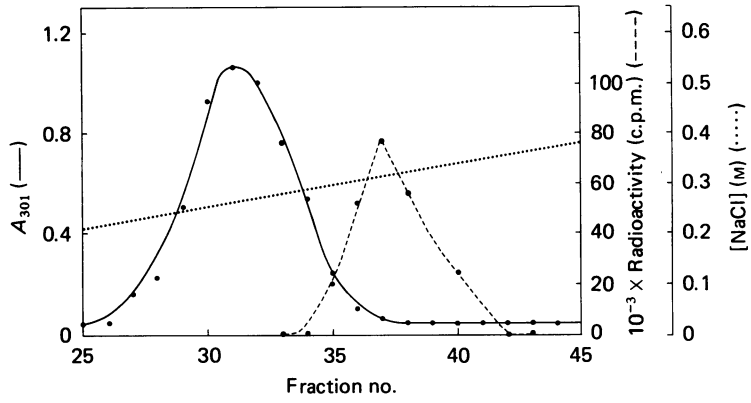

Fig. 2. Separation of H_4MPT and cytoplasmic cofactor by column chromatography

H_4MPT was assayed by u.v. spectrometry. Cytoplasmic cofactor was assayed in the methane-synthesizing system described in the text. Chromatographic procedures and gradient buffer are described in the Materials and methods section.

compound showed a peak at 777 mass units ($M+H$), corresponding to the M_r of 776 for H_4MPT.

H_4MPT is co-eluted with the cytoplasmic cofactor at neutral pH from a DEAE-cellulose anion-exchange column (Sauer *et al.*, 1984). Good separation of cytoplasmic cofactor and H_4MPT can be achieved if the ATS fraction is adjusted to pH 4.5 when applied to the column and a shallow NaCl gradient is used (Fig. 2). At this stage of purification, the cofactor had a slight yellow colour and some yellow fluorescence. This contamination was removed by the second DEAE-Sephadex column. Cytoplasmic cofactor, when pure, is colourless and appears to have no characteristic u.v. absorption spectrum.

In the absence of H_4MPT or CH_3-S-CoM, $^{14}CO_2$ reduction to $^{14}CH_4$ is inhibited by 92% and 96 respectively of control values (Table 2), which suggests

Table 2. The role of tetrahydromethanopterin in methane synthesis

The incubation mixture contained: 25 mM-KCl, 25 mM potassium-phosphate buffer, pH 7.0; 2.5 mM-ATP; 3 mM-Mg^{2+}; 2.5 mM-titaniumcitrate; 0.5 mM-CH_3-S-CoM; 85 μg of H_4MPT; 1.1 mg of membrane protein; and 50 μl of cytoplasmic cofactor (except where indicated otherwise) in a final volume of 0.4 ml. $H^{14}CO_3^-$ (sp. radioactivity 2.7 mCi/mmol, 1.2×10^6 c.p.m.) was added to all samples. MPT was derived from H_4MPT air-oxidized for 40 min. Limit of unlabelled CH_4 detection was 10 nmol. Abbreviation used: n.d., not detected.

Cofactor omitted	Methane production	
	Total (nmol)	Radioactivity (c.p.m.)
None	164	350200
CH_3-S-CoM	n.d.	12500
H_4MPT	38	29500
MPT added instead of H_4MPT	49	6000

Table 3. The role of cytoplasmic cofactor in methane synthesis

The results were obtained when DEAE-Sephadex fractions were assayed for the presence of cytoplasmic cofactor. Cytoplasmic cofactor was limiting in all assays. The conditions of incubation were as in Table 2. $H^{14}CO_3^-$ (sp. radioactivity 3.6 mCi/mmol, 8.0×10^5 c.p.m.) was used. The limit of unlabelled-methane detection was 10 nmol. Abbreviation used: n.d., not detected.

Expt. no.	Cytoplasmic cofactor	[^{14}C]Methane from $H^{14}CO_3^-$ (nmol)	Methane from CH_3-S-CoM (nmol)
1	−	10.0±0.33	n.d.
	+	17.6±0.96	68.4±4.73
2	−	6.8±0.24	n.d.
	+	12.3±0.46	57.7±6.54
3	−	9.2±0.65	n.d.
	+	15.3±0.23	58.3±4.25

that both compounds are intermediate carbon carriers in methane synthesis. When H_4MPT is oxidized to MPT, $^{14}CO_2$ reduction to methane is almost completely inhibited (98%), but the methyl reductase reaction is only partly inhibited (70%). This confirms the proposal that H_4MPT is a CO_2 carrier in methane synthesis (Van Beelen *et al.*, 1984*b*).

There is an absolute requirement for the cytoplasmic cofactor in the reduction of CH_3-S-CoM to methane (Table 3). This requirement is less strict for the reduction of CO_2 to methane. In the absence of cytoplasmic cofactor the rate of CO_2 reduction to methane is decreased by only 42.6%. The membrane fraction apparently contains the cofactor in quantities that are

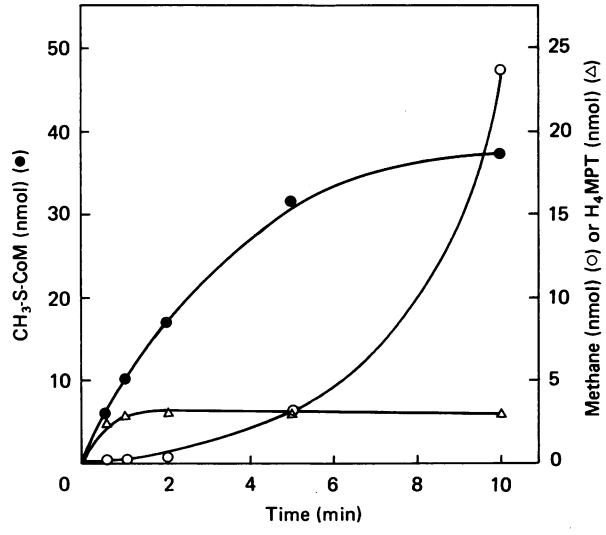

Fig. 3. Kinetics of ^{14}C appearance in H_4MPT, CH_3-S-CoM and methane

The incubation procedure was as described in the text for indicated time intervals. $H^{14}CO_3^-$ (sp. radioactivity 2.7 mCi/mmol, 1.2×10^6 c.p.m.) was added to the incubations. Labelled methane (○), H_4MPT (△) and CH_3-S-CoM (●) were isolated and monitored as described in the Materials and methods section.

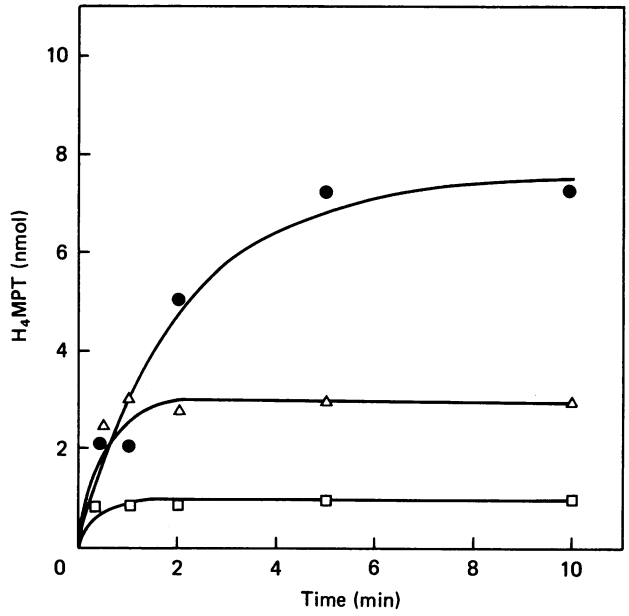

Fig. 4. Appearance of ^{14}C in H_4MPT with time

The incubation procedure was as in Fig. 3. The reaction mixture was incubated without H_4MPT (□), with the complete system (△) and without CH_3-S-CoM (●). The labelled compounds were isolated and monitored for radioactivity as described in the text.

sufficient to convert measurable amounts of CO_2 into methane but which are not adequate to release detectable amounts (i.e. > 10 nmol) of methane from added CH_3-S-CoM.

The short-term kinetics of ^{14}C incorporation into H_4MPT (methylene and methyl derivatives) and CH_3-S-CoM are consistent with their role as intermediate carbon carriers in methanogenesis (Fig. 3). The H_4MPT fraction was rapidly labelled and reached a plateau at ≃ 3 nmol of [^{14}C]methylene-H_4MPT. [^{14}C]CH_3-S-CoM was also rapidly labelled and approached plateau values after 5 min. [^{14}C]Methane production showed a definite lag and did not begin for several minutes after CH_3-S-CoM became labelled.

The H_4MPT fraction accumulated label when CH_3-S-CoM was omitted from the incubation mixture (Fig. 4). This is consistent with the role of HS-CoM as the terminal carbon carrier in methane production. When H_4MPT was omitted from the incubation mixture, endogenous H_4MPT still accumulated small, but easily measurable, amounts of radioactivity (Fig. 4). This shows that some H_4MPT is tightly associated with the membrane fraction. This was also indicated by the results in Fig. 5. There was still significant accumulation of ^{14}C in the CH_3-S-CoM fraction, even with the omission of added H_4MPT from the incubation mixture. Some [^{14}C]CH_3-S-CoM was formed when HS-CoM was substituted for CH_3-S-CoM. No [^{14}C]CH_3-S-CoM was formed when neither CH_3-S-CoM nor HS-CoM was added.

The *M. thermoautotrophicum* membrane fraction reduced formaldehyde to methane considerably faster than CO_2 (Table 4). Even with small quantities of [^{14}C]formaldehyde (4.5 nmol), 16% conversion into methane was obtained. [^{14}C]Methylene-H_4MPT or

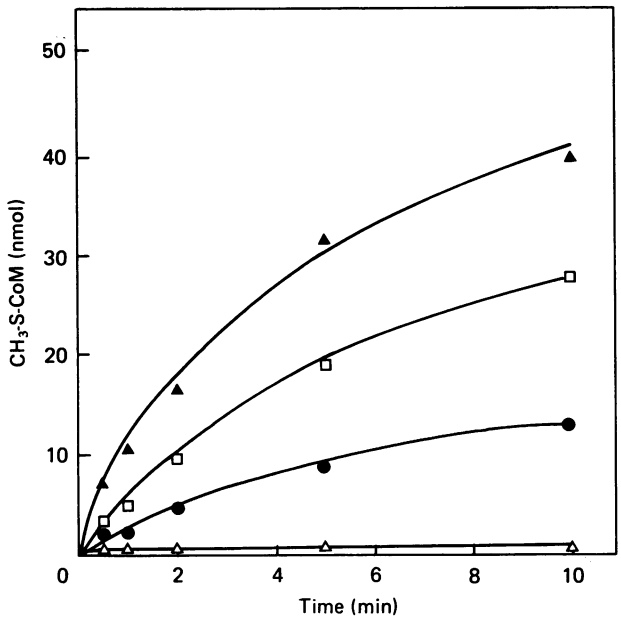

Fig. 5. Appearance of [^{14}C]CH_3-S-CoM with time

The reaction mixture was incubated with CH_3-S-CoM and H_4MPT added (▲), with H_4MPT omitted (□), with HS-CoM added instead of CH_3-S-CoM (●), and with CH_3-S-CoM and H_4MPT omitted (△).

[^{14}C]formaldehyde plus added H_4MPT showed good rates of conversion into methane, which also indicates that the methylene-H_4MPT derivative is a precursor in methanogenesis. Under the conditions of incubation, formaldehyde and H_4MPT would readily condense to form the methylene derivative of H_4MPT.

As reported previously (Sauer *et al.*, 1979), the surfactant deoxycholate inhibited methane synthesis (Table 5) at concentrations of 0.125 and 0.25%. With a view to solubilizing the enzymes of methane synthesis, other detergents were tested, i.e. the zwitterionic surfactants. These are 3-(alkyldimethylammonio)-propane-1-sulphonates, in which the alkyl chain length is 8–16 carbon atoms. The CMC of these sulphobetaines decreases logarithmically as a function of increasing chain length (Gonenne & Ernst, 1978). The sulphobetaines of shorter chain lengths ($n < 12$) solubilize membrane proteins at concentrations below the CMC, whereas those with an alkyl chain length of 12 (CMC = 0.12%) or longer are most effective above the CMC. Almost complete inhibition of methane synthesis was noted with a chain length of 12 carbon atoms (Table 5), with notably less inhibition when the alkyl chain was either shorter or longer.

Table 4. Conversion of 5,10-methylene-5,6,7,8-H_4MPT into methane by membrane fraction

The incubation mixture was as in Table 2, except where indicated otherwise. Membrane protein (1.0 mg) was added to the mixtures. Where indicated, H_4MPT (72 μg) was added. [^{14}C]Methylene-H_4MPT (sp. radioactivity 10 mCi/mmol, 248560 c.p.m.), formaldehyde (400 nmol), [^{14}C]formaldehyde (sp. radioactivity 10 mCi/mmol, 1.0×10^5 c.p.m.) or $H^{14}CO_3^-$ (sp. radioactivity 10 mCi/mol, 4.3×10^6 c.p.m.) was added as indicated.

Substrate	Total methane production (nmol)	Radioactive precursor converted (%)
Formaldehyde + $^{14}CO_2$ + H_4MPT	412	5.2
[^{14}C]Formaldehyde	60	16.0
[^{14}C]Formaldehyde + H_4MPT	161	26.0
[^{14}C]Methylene-H_4MPT	130	25.8

Table 5. Inhibition of methane synthesis by surfactants

The conditions of incubation were as described in Table 2, except that in Expt. 1 the CH_3-S-CoM concentration was increased to 1.54 mM. $H^{14}CO_3$ (sp. radioactivity 3.64 mCi/mmol, 1.6×10^6 c.p.m.) was used. The numbering system of the sulphobetaine detergents designates the length of the alkyl chain. Abbreviation used: n.d., not detected.

Expt. no.	Inhibitor	Methane produced (nmol)	[^{14}C]Methane produced (c.p.m.)
1	None	355	87900
	Deoxycholate (0.25%)	n.d.	100
2	None	113	73400
	Deoxycholate (0.125%)	n.d.	3100
3	None	158	119260
	Detergent 3–8 (0.25%)	57	19310
	Detergent 3–12 (0.25%)	n.d.	750
	Detergent 3–16 (0.25%)	28	10760

DISCUSSION

Methanopterin is a 2-amino-4-hydroxy-7-methylpteridine derivative with a side chain that consists of an ethyl group esterified to aniline, tetrahydroxypentane, α-ribofuranose, phosphate and α-hydroxyglutaric acid (Van Beelen *et al.*, 1984*a*). The structure was also confirmed by ^{13}C and ^{1}H n.m.r. in the present work.

H_4MPT becomes a one-carbon carrier with the formation of 5,10-methenyl-(or methylene-)5,6,7,8-tetrahydromethanopterin (Van Beelen *et al.*, 1984*b*), and the evidence indicates that CH_3-H_4MPT is formed via a series of reductive steps. This is reminiscent of one-carbon reductions carried out by tetrahydrofolate derivatives that have been shown to synthesize methane but at very low rates (Sauer *et al.*, 1977). In agreement with the results by Escalante-Semerena *et al.* (1984*b*), those presented here show that chemically prepared methylene-H_4MPT readily forms methane when incubated with enzyme in an atmosphere of H_2.

Recent results indicate that CO_2 activation and reduction to the formyl level of oxidation are carried out by a cofactor with the trivial name methanofuran and which transfers a formyl group to H_4MPT (Leigh *et al.*, 1985). This is in contrast with the synthesis of formyltetrahydrofolate by most prokaryotes and eukaryotes. For that reaction the relevant enzymes, i.e. formyltetrahydrofolate synthetase, methenyltetrahydrofolate cyclohydrolase, and methylenetetrahydrofolate dehydrogenase, have been well characterized, isolated and purified (Paukert *et al.*, 1976).

A pathway has been proposed whereby methenyl-H_4MPT is reduced stepwise to methyl-H_4MPT, followed by a transfer of the methyl group to HS-CoM (Leigh *et al.*, 1985). The kinetics of carbon labelling presented here give direct experimental evidence for the first time that this proposed methyl group transfer from H_4MPT to HS-CoM actually occurs. Evidence for this lies in the observation that labelled carbon from $^{14}CO_2$ appeared first in the H_4MPT fraction, then in CH_3-S-CoM and finally, after a short lag, in methane. When CH_3-S-CoM was left out of the incubation mixture, labelled carbon accumulated in the H_4MPT fraction as expected.

It is now clear that the failure in earlier experiments (Sauer *et al.*, 1980) to demethylate CH_3-S-CoM was due to the omission of the cytoplasmic cofactor from the reaction mixture. As shown in the present experiments, CO_2 reduction to methane by the membrane fraction of *M. thermoautotrophicum* can proceed without added cytoplasmic cofactor, but only at decreased rates. On the other hand CH_3-S-CoM reduction to methane is totally dependent on the addition of cytoplasmic cofactor. It therefore appears that sufficient cytoplasmic cofactor is retained by the membrane fraction to allow CO_2 reduction to methane, but that for CH_3-S-CoM conversion into methane, larger concentrations of this cofactor are required. Tanner (1982) has described a factor, called 'component B', which has properties similar to those of the cytoplasmic cofactor. The u.v. and n.m.r. spectra reported for component B (Tanner, 1982), however, differ significantly from those observed for cytoplasmic cofactor.

With the exception of cytoplasmic cofactor, the structures of the different cofactors of methane synthesis have been characterized. Much less information is available about the enzymes of methane synthesis. Some success was reported by Nagle & Wolfe (1983), who were able partially to resolve and characterize the methyl-coenzyme M methyl reductase system of *M. thermoautotrophicum*. There is, however, little information about the enzyme(s) of CO_2 activation of the enzyme(s) which reduce the H_4MPT derivatives. In order to solubilize the enzymes of the methane-synthesizing complex, we used a series of novel sulphobetaine detergents, which consist of an alkyl chain, a strongly basic quaternary ammonium ion and an acidic sulphonate ion. These detergents have zwitterionic properties over a wide pH range (Gonenne & Ernst, 1978) and, in addition, have the advantage of being good solubilizing agents that generally do not inactivate either membrane-bound or soluble enzymes. Nevertheless, these sulphobetaines inhibited methane synthesis with maximum inhibition with the 12-carbon alkyl-chain detergent. It would probably be easier to isolate individual enzymes rather than attempt to solubilize the entire methane-synthesizing complex. These types of studies will be greatly facilitated by the availability of the newly isolated cofactors.

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