

Tetrahydromethanopterin, a coenzyme involved in autotrophic acetyl coenzyme A synthesis from 2 CO₂ in *Methanobacterium*

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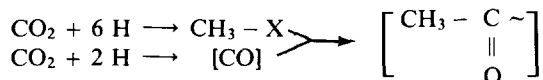
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Methanobacterium thermoautotrophicum, a methane forming archaeobacterium, grows autotrophically by synthesizing activated acetic acid from 2 CO₂. It is demonstrated in vitro that the methyl group of acetate is derived from methenyl tetrahydromethanopterin, which is known to be a one-carbon carrying coenzyme in CO₂ reduction to methane. The direct acetate precursors are suggested to be methyl tetrahydromethanopterin ('activated methanol') and 'activated carbon monoxide'.

Methanobacterium Methanopterin Autotrophy Acetyl-CoA synthesis Archaeobacteria Pteridin

1. INTRODUCTION

Methanobacterium thermoautotrophicum is a methane-forming archaeobacterium which grows on H₂ and CO₂ as sole energy and carbon sources [1,2]. This organism as well as other anaerobic autotrophic bacteria assimilates cell carbon from CO₂ primarily into acetyl-CoA via a novel autotrophic pathway. The principle of this process, which has extensively been studied in the heterotrophic *Clostridium thermoaceticum* (reviews [4,5]), is the following:



The carboxyl of acetate is derived by a carbonylation type of reaction from 'activated carbon monoxide' [CO] via the nickel enzyme carbon monoxide dehydrogenase [6–9]. The synthesis of the methyl of acetate in *Methanobacterium* is

linked to CO₂ reduction to methane [10–12], probably by sharing common one-carbon intermediates [13]. Methanogenesis from CO₂ involves coenzyme-bound one-carbon units linked to methanofurane (activated CO₂, possibly activated formate) [14], methanopterin (activated formate, formaldehyde, methanol) [15], and coenzyme M (activated methanol) [16]. Methyl coenzyme M reduction leads to methane; methyl coenzyme M has been shown not to be precursor of the methyl group of acetate [13].

Here, [methenyl-¹⁴C]tetrahydromethanopterin (activated formate) was prepared according to [18,19] and tested as to whether it could serve as a precursor of acetate. Label from this compound was not only readily converted into methane, but was also specifically incorporated into the methyl of acetate.

2. MATERIALS AND METHODS

2.1. Cells and cell extract

M. thermoautotrophicum Marburg strain (DSM 2133) was grown at 65°C on mineral medium under gassing with 80% H₂, 20% CO₂, 0.1% H₂S

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Dedicated to Professor Decker on the occasion of his 60th birthday

as the energy, carbon, and sulfur sources. Cultivation in large scale, harvesting of cells, growth determination, and anaerobic preparation of cell extract was as described [12].

2.2. *In vitro* assay of [^{14}C]acetate formation

The *in vitro* assay (550 μl , 60°C) routinely contained: 110 mM Pipes-Na buffer (pH 6.75); 2 mM ATP; 7 mM MgCl_2 ; 3 mM methyl coenzyme M; 4 mM DTE; 0.2 mM CoA; 0.7 mM $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$; 100 μl extract (4 mg protein); $^{14}\text{CO}_2$ and [^{14}C -methenyl]tetrahydromethanopterin, respectively. Gas phase (5 ml) contained 80% H_2 /20% CO_2 and 100% H_2 , respectively. The assay was stopped after 30 min, [^{14}C]acetyl-CoA was hydrolyzed, and [^{14}C]acetate and [^{14}C]formate were isolated [12]. [^{14}C]Acetate was chemically degraded by Schmidt degradation [12]. Methane was determined by gas chromatography, protein by the Biuret method. ^{14}C was determined by liquid scintillation counting. Tetrahydromethanopterin was enzymatically (0.5 μl cell extract) determined in 1 ml assay [18,19]; the reaction was started with 0.3 μmol formaldehyde.

2.3. Preparation of tetrahydromethanopterin (H_4MPT)

H_4MPT was isolated under anoxic, acidic conditions from boiled cell extract of 40–120 g fresh cells essentially as described [18,19]; before HPLC and instead of desalting by Sephadex G 10 after HPLC, the pooled H_4MPT -containing fraction was chromatographed on XAD-4 Amberlite, 0.1–0.2 mm, in 2 mM HCOOH with a 0–100% linear methanol gradient (25% yield).

2.4. Preparation of [^{14}C -methenyl] H_4MPT

H_4MPT (1.1 μmol) was enzymatically (2 μl cell extract) converted [19] with 2.2 μmol [^{14}C]formaldehyde (22×10^6 dpm) into [^{14}C -methenyl]- H_4MPT (8×10^6 dpm), which was separated from excess $^{14}\text{CH}_2\text{O}$ by XAD-4 chromatography (see above). Controls run in parallel without H_4MPT contained no radioactivity in the corresponding fractions.

3. RESULTS

From *M. thermoautotrophicum* H_4MPT was isolated by anionic exchange, adsorption and

HPLC (fig.1). From purified H_4MPT and [^{14}C]formaldehyde, [^{14}C -methenyl] H_4MPT was prepared. The ^{14}C -labelled compound was radiochemically pure and was clearly separated from [^{14}C]formaldehyde. This is important because cell extracts readily converted [^{14}C]formaldehyde into CH_4 and the methyl of acetate. The UV spectra of H_4MPT and methenyl H_4MPT are shown in fig.2 (cf. [18]).

With cell extracts methane and acetate synthesis from $^{14}\text{CO}_2$ proceeded at 160 $\text{nmol min}^{-1} \cdot \text{mg protein}^{-1}$ and 1 $\text{nmol min}^{-1} \cdot \text{mg protein}^{-1}$, respectively (table 1). In addition to CO_2 the assay contained 3 mM methyl coenzyme M as one-carbon precursors, and hydrogen as reductant. Label in [^{14}C]acetate was equally distributed (table 2).

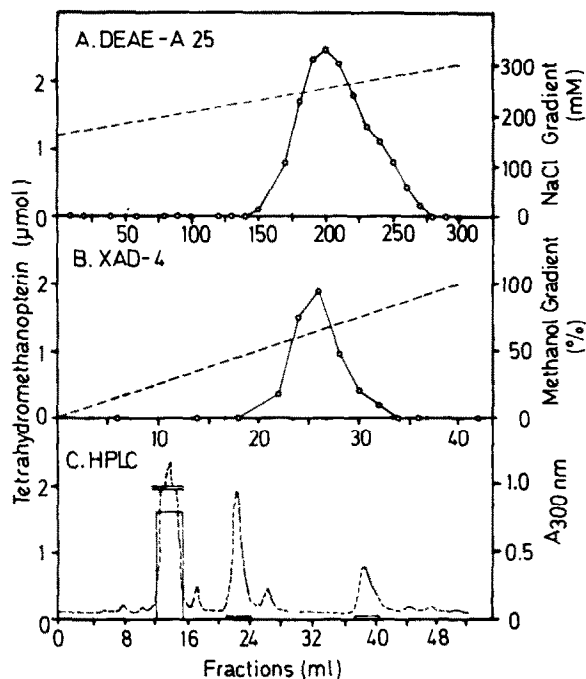


Fig.1. Preparation of tetrahydromethanopterin from *M. thermoautotrophicum* ([18] modified). (A) DEAE A-25 anion exchange chromatography at pH 4.8, 160–300 mM NaCl (---). (B) XAD-4 adsorption chromatography at pH 4.5, 0–100% methanol (---). (C) Preparative HPLC on reversed phase C-18, isocratic at pH 3.0, 17% methanol; (---) absorption at 300 nm. Only fractions 12–15 contained H_4MPT (—). The amounts refer to material from boiled cell extract from 40 g cells (fresh weight).

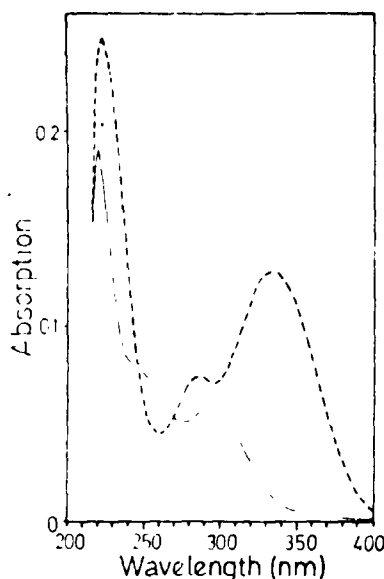


Fig.2. UV spectra of tetrahydromethanopterin (—) and *N*5,*N*10-methenyltetrahydromethanopterin (---) in 20 mM phosphate buffer (pH 7.0) under anoxic conditions; (---) was prepared enzymatically from (—) and formaldehyde.

Under identical conditions, except for CO_2 being unlabelled, [^{14}C -methenyl] H_4MPT was readily metabolized. Most of the label (98%) was converted into CH_4 ; the acid-stable fraction contained [^{14}C]acetate and little [^{14}C]formate (table 1). The ratio of ^{14}C incorporated into CH_4 and acetate was 250:1. [^{14}C]Acetate was isolated and degraded. It contained virtually all the label from [^{14}C -methenyl] H_4MPT in the methyl group (table 2). Similar results were obtained when only endogenous CO_2 and methyl coenzyme M were available as carbon sources, in addition to [^{14}C -

Table 1

Conversion of ^{14}C from $^{14}\text{CO}_2$ and [^{14}C -methenyl]tetrahydromethanopterin into $^{14}\text{CH}_4$ and [^{14}C]acetyl-CoA (isolated as [^{14}C]acetate) by cell extract of *M. thermoautotrophicum*

Assay containing	^{14}C added (dpm)	Methane formed (μmol)	^{14}C remaining in solution (dpm)	^{14}C extractable with ether at acidic pH (dpm)	^{14}C in acetate (dpm)	^{14}C in formate (dpm)
$^{14}\text{CO}_2/\text{H}_2$	2.2×10^7	20.1	7.1×10^5	2.4×10^5	8.8×10^4	7.1×10^4
[^{14}C -methenyl]Tetrahydromethanopterin (76 nmol)						
CO_2/H_2	8.3×10^5	16.2	1.5×10^4	2.6×10^3	2.2×10^3	2.5×10^2
[^{14}C -methenyl]Tetrahydromethanopterin (76 nmol) H_2	8.3×10^5	1.5	2.1×10^5	6.8×10^3	4.7×10^3	8.0×10^2

Assay: standard assay containing 8 mM methyl coenzyme M and 100 μl extract; 10 μCi $^{14}\text{CO}_2$ and 0.38 μCi [^{14}C -methenyl]tetrahydromethanopterin, respectively. Incubation at 60°C for 30 min

Table 2

Label distribution in [^{14}C]acetate formed by cell extracts of *M. thermoautotrophicum* from $^{14}\text{CO}_2$ and [^{14}C -methenyl]tetrahydromethanopterin. For control, authentic [$\text{U-}^{14}\text{C}$]acetate and [$2\text{-}^{14}\text{C}$]acetate were degraded by the same procedure (Schmidt degradation)

[^{14}C]Acetate from	^{14}C added (dpm)	^{14}C recovered from COOH group		^{14}C recovered from CH_3 group	
		dpm	%	dpm	%
$^{14}\text{CO}_2$	6370	3130	49	2920	46
[^{14}C -methenyl]Tetrahydromethanopterin	4950	80	2	3990	81
Control: [$\text{U-}^{14}\text{C}$]acetate standard	26150	13175	50	12535	48
Control: [$2\text{-}^{14}\text{C}$]acetate standard	22140	70	0.3	21600	98

methenyl] H_4MPT , and hydrogen served as electron donor (table 1).

4. DISCUSSION

It was shown that carbon from [methenyl- ^{14}C]-tetrahydromethanopterin was incorporated into methane and into the methyl of acetate as close to the same ratio as was $^{14}\text{CO}_2$ incorporated into these compounds in vitro. This indicates that energy metabolism (CO_2 reduction to methane) and autotrophic fixation of CO_2 into the methyl of acetate (representing approximately 30% of cell carbon) have in common the path of CO_2 reduction to methyl tetrahydromethanopterin; besides *Methanobacterium*, this probably applies also to other autotrophic methanogens. In *Methanobacterium*, folate appears to be lacking [21]. Its pteridin counterpart, methanopterin [15], was shown to act as one-carbon carrier in the process of CO_2 reduction to methane [15,18,20]; [^{14}C -methenyl]tetrahydromethanopterin was first noticed as yellow fluorescent compound in $^{14}\text{CO}_2$ -pulse-labelled cells [22].

Since the early days of activated acetic acid research (for a classic see [23]), the role of acetyl-CoA in metabolism seemed to be settled. From the work with the heterotrophic *C. thermoaceticum* it was suggested that acetate synthesis from CO_2 may prove to be an example of a heterotrophic as well as autotrophic CO_2 fixation [24]. In Clostridia, the one-carbon carrier in CO_2 reduction to the methyl level is tetrahydrofolate (for literature see [4,5]). One-carbon compound synthesis from CO_2 is required in acetogenic bacteria as precursor for the methyl of acetate [4,5] in homoacetate fermentation, or as biosynthetic one-carbon precursor [25]. In recent years it became evident that the principle of this path is realized in anaerobic chemolithoautotrophs to synthesize acetyl CoA only from CO_2 . Total synthesis of acetyl-CoA occurs in quite distantly related groups of procaryotes, in methanogenic bacteria (archaeobacteria) [12,13], acetogenic bacteria (gram (+) eubacteria) [5,26], and sulfate-reducing bacteria (gram (+) and gram (-) eubacteria) [27]. This wide distribution in anaerobes is considered as indication for a primitive or ancient path.

Our findings have several implications: (i) in future work the process of acetyl-CoA synthesis

can be studied from simpler precursors, such as [^{14}C -methyl]tetrahydromethanopterin and carbon monoxide; electron transfer reactions are no longer required. (ii) Methanogenic, acetogenic, and sulfate-reducing bacteria are of ecological and economical interest (sewage treatment, acetate production, corrosion); basic knowledge of their metabolism is still rudimentary. (iii) The demonstration of the role of pteridines in acetate synthesis in *Methanobacterium* adds decisive information how autotrophs assimilate CO_2 via this non-Calvin pathway.

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REFERENCES

- [1] Zeikus, J.G. and Wolfe, R.S. (1972) *J. Bacteriol.* 109, 707-713.
- [2] Balch, W.E., Fox, G.E., Magrum, L.J., Woese, C.R. and Wolfe, R.S. (1979) *Microbiol. Rev.* 43, 260-296.
- [3] Fuchs, G. and Stupperich, E. (1983) *Physiol. Vég.* 21, 845-854.
- [4] Ljungdahl, L. and Wood, H.G. (1982) in: *B₁₂* (Dolphin, D. ed.) pp.165-201, Wiley, New York.
- [5] Wood, H.G., Drake, H.L. and Hu, S.I. (1982) *Proc. Biochem. Symp.* 28-56, *Ann. Rev.*, Pasadena.
- [6] Hu, S.I., Drake, H.L. and Wood, H.G. (1982) *J. Bacteriol.* 149, 440-448.
- [7] Stupperich, E., Hammel, K.E., Fuchs, G. and Thauer, R.K. (1984) *FEBS Lett.* 152, 21-23.
- [8] Eikmanns, B., Fuchs, G. and Thauer, R.K. (1984) *Eur. J. Biochem.*, in press.
- [9] Diekert, G., Fuchs, G. and Thauer, R.K. (1984) in: *Microbial Gas Metabolism* (Poole, R.K. ed.) Academic Press, in press.
- [10] Kenealy, W.R. and Zeikus, J.G. (1982) *J. Bacteriol.* 151, 932-941.
- [11] Stupperich, E. and Fuchs, G. (1983) *FEBS Lett.* 156, 345-348.
- [12] Stupperich, E. and Fuchs, G. (1984) *Arch. Microbiol.* 139, 8-13.
- [13] Stupperich, E. and Fuchs, G. (1984) *Arch. Microbiol.* 139, 14-20.

- [14] Leigh, J.A., Rinehart, K.L. and Wolfe, R.S. (1984) *J. Am. Chem. Soc.* 106, 3636–3639.
- [15] VanBeelen, P., Stassen, A.P.M., Bosch, J.W.G., Vogels, G.D., Guijt, W. and Haasnoot, C.A.G. (1984) *Eur. J. Biochem.* 138, 563–571.
- [16] Taylor, C.D. and Wolfe, R.S. (1974) *J. Biol. Chem.* 249, 4879–4885.
- [17] Gunsalus, R.P. and Wolfe, R.S. (1980) *J. Biol. Chem.* 255, 1891–1895.
- [18] Escalante-Semerena, J.C., Rinehart, K.L. and Wolfe, R.S. (1984) *J. Biol. Chem.* 259, 9447–9455.
- [19] Escalante-Semerena, J.C. (1983) Dissertation, University of Illinois, Urbana, IL.
- [20] VanBeelen, P., DeCock, R.M., Guijt, W., Haasnoot, C.A.G. and Vogels, G.D. (1984) *FEMS Microbiol. Lett.* 21, 159–163.
- [21] Leigh, J.A. (1983) *Appl. Environmental Microbiol.* 45, 800–803.
- [22] Daniels, L. and Zeikus, J.G. (1978) *J. Bacteriol.* 136, 75–84.
- [23] Decker, K. (1959) *Die aktivierte Essigsäure*. Ferdinand Enke Publisher, Stuttgart.
- [24] Wood, H.G. and Utter, M.F. (1965) in: *Essays in Biochemistry* (Campbell, B.N. and Greville, G.D. eds) vol.1, pp.1–27.
- [25] Jungermann, K., Thauer, R.K. and Decker, R.K. (1968) *Eur. J. Biochem.* 3, 351–359.
- [26] Eden, G. and Fuchs, G. (1982) *Arch. Microbiol.* 133, 66–74.
- [27] Jansen, K., Thauer, R.K., Widdel, F. and Fuchs, G. (1984) *Arch. Microbiol.* 138, 257–262.