Tetrahydromethanopterin, a coenzyme involved in autotrophic acetyl coenzyme A synthesis from 2 CO_2 in *Methanobacterium*

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Methanobacterium thermoautotrophicum, a methane forming archaebacterium, grows autotrophically by synthesizing activated acetic acid from 2 CO_2. 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 CO_2 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* *Archaebacteria* *Pteridin*

1. INTRODUCTION

Methanobacterium thermoautotrophicum is a methane-forming archaebacterium which grows on H_2 and CO_2 as sole energy and carbon sources [1,2]. This organism as well as other anaerobic autotrophic bacteria assimilates cell carbon from CO_2 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:

$$\left.\begin{array}{l} CO_2 + 6\,H \longrightarrow CH_3 - X \\ CO_2 + 2\,H \longrightarrow [CO] \end{array}\right\rangle \longrightarrow \left[\begin{array}{l} CH_3 - C \sim \\ \quad\quad\;\; \| \\ \quad\quad\;\; O \end{array}\right]$$

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 CO_2 reduction to methane [10–12], probably by sharing common one-carbon intermediates [13]. Methanogenesis from CO_2 involves coenzyme-bound one-carbon units linked to methanofurane (activated CO_2, 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-^{14}C]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% H_2, 20% CO_2, 0.1% H_2S

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

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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 $(NH_4)_2Fe(SO_4)_2$; 100 μl extract (4 mg protein); $^{14}CO_2$ 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_4-MPT (8 × 10^6 dpm), which was separated from excess $^{14}CH_2O$ 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 $^{14}CO_2$ proceeded at 160 nmol $min^{-1} \cdot mg\ protein^{-1}$ and 1 nmol $min^{-1} \cdot mg\ protein^{-1}$, 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).

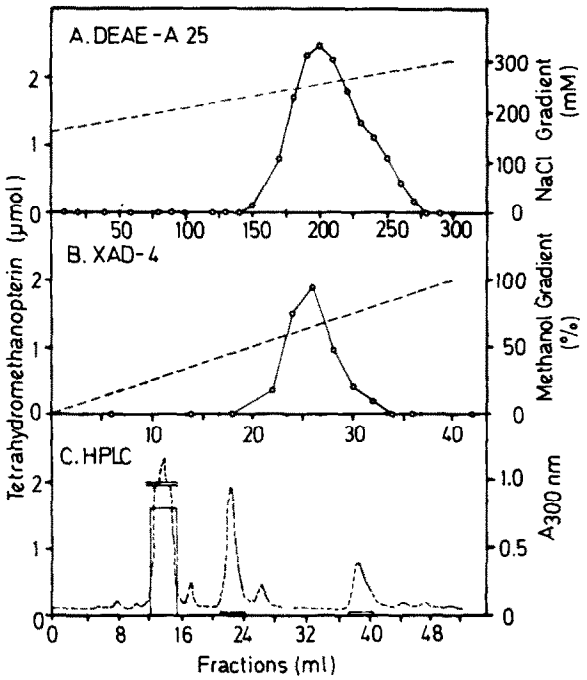

Fig.1. Preparation of tetrahydromethanopterin from *M. thermoautotrophicum* ([18] modified). (A) DEAE A-25 anion exchange chromatography at pH 4.8, 160–300 mM NaCl (– – –). (B) XAD-4 adsorption chromatography at pH 4.5, 0–100% methanol (– – –). (C) Preparative HPLC on reversed phase C-18, isocratic at 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).

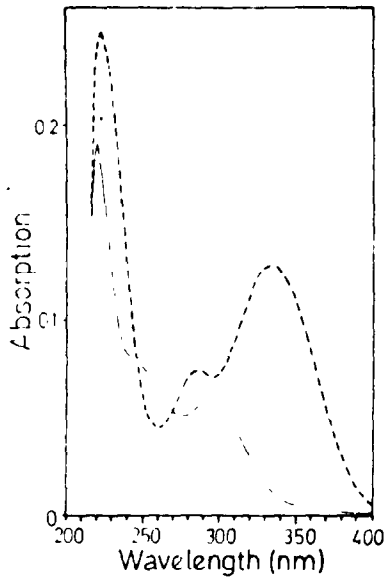

Fig.2. UV spectra of tetrahydromethanopterin (——) and *N*5,*N*10-methenyltetrahydromethanopterin (– – –) in 20 mM phosphate buffer (pH 7.0) under anoxic conditions; (– – –) was prepared enzymatically from (——) and formaldehyde.

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 $^{14}CO_2$ and [^{14}C-methenyl]tetrahydromethanopterin into $^{14}CH_4$ 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)
$^{14}CO_2/H_2$ [^{14}C-methenyl]Tetrahydromethanopterin (76 nmol)	2.2×10^7	20.1	7.1×10^5	2.4×10^5	8.8×10^4	7.1×10^4
CO_2/H_2 [^{14}C-methenyl]Tetrahydromethanopterin (76 nmol)	8.3×10^5	16.2	1.5×10^4	2.6×10^3	2.2×10^3	2.5×10^2
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 $^{14}CO_2$ 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 $^{14}CO_2$ and [^{14}C-methenyl]tetrahydromethanopterin. For control, authentic [U-^{14}C]acetate and [2-^{14}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	%
$^{14}CO_2$	6370	3130	49	2920	46
[^{14}C-methenyl]Tetrahydromethanopterin	4950	80	2	3990	81
Control: [U-^{14}C]acetate standard	26150	13175	50	12535	48
Control: [2-^{14}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 $^{14}CO_2$ 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 $^{14}CO_2$-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 (archaebacteria) [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: B_{12} (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.