Methanogenesis Involving a Novel Carrier of \( \text{C}_1 \) Compounds in \( \text{Methanogenium tationis} \)

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The pathway of \( \text{CO}_2 \) reduction to methane in \( \text{Methanogenium tationis} \) and \( \text{Methanogenium thermophilicum} \) is similar to that observed in other methanogens. In \( \text{M. tationis} \) a novel pterin, tatipterin, is present. This pterin appears to be a structural and functional analog of methanopterin and sarcinapterin. Folate could not substitute for tatipterin.

The biochemistry of methanogenesis is characterized by the involvement of unique coenzymes (10). Previous studies showed that methanogenesis proceeds along a common pathway in different methanogens (8). Recently it was found that in \( \text{Methanogenium tationis} \) and \( \text{Methanogenium thermophilicum} \) neither methanopterin nor sarcinapterin was present (4). A novel pterin has been found in \( \text{M. tationis} \), and its structure has been elucidated (Fig. 1) (P. C. Raemakers-Franken, F. G. J. Voncken, J. Korteland, J. T. Keltjens, C. van der Drift, and G. D. Vogels, BioFactors, in press).

In this study, we present results indicating that the pathway of methanogenesis in \( \text{M. tationis} \) and \( \text{M. thermophilicum} \) is similar to that in other methanogens and that the novel pterin, tatipterin, from \( \text{M. tationis} \) has the same function as methanopterin and sarcinapterin.

\( \text{M. thermophilicum} \) (DSM 2373) and \( \text{M. tationis} \) (DSM 2702) were cultured in a medium described by Zabel et al. (16, 17) on \( \text{H}_2-\text{CO}_2 \) (80:20, vol/vol) or 60 mM formate in 100-ml serum bottles containing 40 ml of medium or in 12-liter fermentors with continuous gas flow (6.4 liters/min).

\( \text{Methanobacterium thermautotrophicum} \) â€” A (DSM 1053) was cultured on \( \text{H}_2-\text{CO}_2 \) by the method of Schönheit et al. (14). Cells were harvested by continuous centrifugation and stored at \(-70^\circ\text{C}\) under an \( \text{N}_2 \) atmosphere.

Cell extracts from \( \text{Methanobacterium thermautotrophicum} \) â€” A were prepared as described before (11). Cell extracts from \( \text{M. tationis} \) and \( \text{M. thermophilicum} \) were prepared by suspending cells in an equal volume of 100 mM TES buffer [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] and treating them with a 150-W sonifier (Branson Sonic Power Co., Danbury, Conn.) at maximum power for 150 and 180 s, respectively, at intervals of 5 s. Cell debris was removed by centrifugation for 30 min at 30,000 \( \times \) g. The supernatant was stored at \(-70^\circ\text{C}\) under an \( \text{N}_2 \) atmosphere.

Cell extracts were made cofactor-free by filtration over an Amicon PM-30 ultrafilter with 100 mM TES buffer (pH 7.0). Boiled cell extracts (BCE) were prepared as described by Keltjens et al. (9). All assays were performed under anaerobic conditions at 40°C, 55°C, or 60°C by using cell extracts from \( \text{M. tationis} \), \( \text{M. thermophilicum} \), and \( \text{Methanobacterium thermautotrophicum} \), respectively.

Cell extracts from \( \text{M. tationis} \) and \( \text{M. thermophilicum} \) were tested for their ability to produce methane from different \( \text{C}_1 \) substrates. The standard mixture for this assay contained 12 \( \mu \text{mol} \) of TES buffer (pH 7.0), 0.5 \( \mu \text{mol} \) of ATP, 5 \( \mu \text{mol} \) of MgCl\(_2\), and 75 \( \mu \text{mol} \) of cell extract (2 mg of protein) in a total volume of 200 \( \mu \text{l} \). Conversion of \( \text{CO}_2 \) as a \( \text{C}_1 \) substrate was measured under an \( \text{H}_2-\text{CO}_2 \) atmosphere (80:20, vol/vol). Conversion of formaldehyde was measured by adding 0.1 \( \mu \text{mol} \) of formaldehyde to the standard mixture under an \( \text{N}_2 \) or \( \text{H}_2 \) atmosphere. Conversion of methylcoenzyme M was measured by adding 0.1 \( \mu \text{mol} \) of methylcoenzyme M to the standard mixture under an \( \text{H}_2 \) or \( \text{H}_2-\text{CO}_2 \) (80:20, vol/vol) atmosphere. The assays were initiated by incubation at the temperatures indicated above. Methane was quantified by gas chromatographic analysis (7).

To test cross-reactivity between the enzyme system of \( \text{Methanobacterium thermautotrophicum} \) and the cofactors of the \( \text{Methanogenium} \) species, cofactor-free cell extract from \( \text{Methanobacterium thermautotrophicum} \) was tested for the ability to produce methane from formaldehyde as a \( \text{C}_1 \) substrate under an \( \text{H}_2 \) atmosphere in a standard assay mixture supplemented with 50 \( \mu \text{g} \) of BCE from \( \text{Methanogenium} \) species.

To determine the effect of a specific inhibitor on methanogenesis, bromoethanesulfonate was added to 40 ml of culture, at a final concentration of 250 \( \mu \text{M} \), and methane production was measured.

Methylenetetrahydrodimermethanopterin dehydrogenase (methylen-H\(_4\)MPT-DH) was measured, as described before (2), by monitoring the conversion of tetrahydromethanopterin (H\(_4\)MPT) to methenyl-H\(_4\)MPT in a mixture containing 180 \( \mu \text{mol} \) of potassium phosphate buffer (pH 6.0), 50 \( \mu \text{mol} \) of H\(_2\)MPT, 86 \( \mu \text{mol} \) of coenzyme F\(_{440}\), 10 \( \mu \text{mol} \) of formaldehyde, and 20 \( \mu \text{mol} \) of cell extract (5 \( \mu \text{g} \) of protein) in a final volume of 2 ml.

Methylen-H\(_4\)MPT reductase (methylene-H\(_4\)MPT-RD) activity was measured by monitoring the formation of methenyl-H\(_4\)MPT from methyl-H\(_4\)MPT in a mixture as described above for the methylen-H\(_4\)MPT-DH assay, except that 50 \( \mu \text{g} \) of protein was used and the reaction was started by adding 50 \( \mu \text{mol} \) of methyl-H\(_4\)MPT instead of H\(_2\)MPT. The formation of methenyl-H\(_4\)MPT and the reduction of coenzyme F\(_{440}\) were monitored spectrophotometrically at 335 and 401 nm, respectively.

Methylenetetrahydrofolate dehydrogenase (methylene-THF-DH) and methylenetetrahydrofolate reductase (methylene-THF-RD) activities were measured in mixtures as described above for the measurement of methylen-H\(_4\)MPT-DH and methylene-H\(_4\)MPT-RD activities, with tetrahydrofolate derivatives instead of H\(_4\)MPT derivatives. Aside from

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coenzyme F\(_{420}\). NADP was tested as a potential electron acceptor at the same concentration as coenzyme F\(_{420}\).

Formate dehydrogenase was measured by the method of Schauer and Ferry (13). Carbon monoxide dehydrogenase (CO-DH) was measured by the method of Krzycki and Zeikus (12), except that Na\(_2\)S\(_2\)O\(_4\) was replaced by 2 mM dithiothreitol.

Recently we reported the presence of a novel pterin in \textit{M. tationis} (Raemakers-Franken et al., in press). Here we report cross-reactivity between enzymes of \textit{Methanobacterium thermoautotrophicum} and pterins of Methanogenium species. Cofactor-free extract from \textit{Methanobacterium thermoautotrophicum} was shown to convert formaldehyde to methane if BCE from \textit{Methanogenium} species was added, with an efficiency of about 20% with respect to addition of BCE from \textit{Methanobacterium thermoautotrophicum}. Thus, the tatioppterin derivatives present in BCE from \textit{Methanogenium} species are recognized by the enzymes of \textit{Methanobacterium thermoautotrophicum}. The occurrence of cross-reactivity was further demonstrated by our observation that methylene-H\(_4\)MPT-DH activity could be measured in cell extracts of \textit{M. tationis} and \textit{M. thermophilicum} (8.0 and 7.1 \(\mu\)mol/min per mg of protein, respectively) if H\(_4\)MPT (isolated from \textit{Methanobacterium thermoautotrophicum}) was added. If H\(_4\)MPT was omitted, to check for an internal C1 carrier, no reaction (activity lower than 0.2 \(\mu\)mol/min per mg of protein) was measured. Likewise, methylene-H\(_4\)MPT-RD activity could be measured in cell extracts of \textit{M. tationis} and \textit{M. thermophilicum} (123 and 76 nmol/min per mg of protein, respectively) if methyl-H\(_4\)MPT isolated from \textit{Methanobacterium thermoautotrophicum} was added. Thus, the enzyme systems of \textit{M. tationis} and \textit{M. thermophilicum} are able to recognize and interconvert H\(_4\)MPT derivatives from \textit{Methanobacterium thermoautotrophicum}. These results indicate that tatioppterin is not only a structural analog but also a functional analog of methanopterin and sarcinapterin.

In eubacteria and eucaryotes, analogous reactions proceed with folate derivatives as C1 carriers and NADP as the electron carrier. Previous work showed that H\(_4\)MPT cannot be replaced by tetrahydrofolate in the methylene-H\(_4\)MPT-DH reaction in \textit{Methanobacterium thermoautotrophicum} (3, 6). Since the chromatoprotein of tatioppterin contains a proton at the 7-position, just as folate derivatives do, we tested whether tetrahydrofolate could substitute for tetrahydrotatioppterin in cell extracts from \textit{M. tationis} and \textit{M. thermophilicum}. Coenzyme F\(_{420}\) or NADP was used as a potential electron carrier. No methylene-THF-DH activity could be measured with the electron carriers tested. Likewise, no methylene-TFH-RD activity could be measured if methylethyltetrahydrofolate was added to cell extracts from \textit{Methanogenium} species in combination with coenzyme F\(_{420}\) or NADP as the electron acceptor. In this assay, activities lower than 0.12 \(\mu\)mol/min per mg of protein could not be measured.

These results indicate that tetrahydrotatioppterin is functionally more related to H\(_4\)MPT and tetrahydrocarpinpterin than to tetrahydrofolute.

Cell extracts from \textit{M. tationis} and \textit{M. thermophilicum} (grown on H\(_2\)-CO\(_2\)) produced methane when incubated with H\(_2\)-CO\(_2\), formaldehyde, or methylcoenzyme M (Table 1). Methylreductase activity could be completely inhibited by the addition of bromoethanesulfonate (250 \(\mu\)M), a specific inhibitor (15), to cells growing on H\(_2\)-CO\(_2\). As expected, formaldehyde conversion was about 50% lower under an N\(_2\) atmosphere than in the presence of H\(_2\) (3). A coupling between the terminal and first steps in the CO\(_2\) reduction to methane (the so-called RFG effect [5]) was not observed. Neither the methane production rate (Table 1) nor the yield of methane obtained increased when methylcoenzyme M was added under an H\(_2\)-CO\(_2\) atmosphere as compared to methylcoenzyme M addition under an H\(_2\) atmosphere.

Because \textit{M. tationis} and \textit{M. thermophilicum} are heterotrophs, which grow on H\(_2\)-CO\(_2\) or formalate as an energy source but require acetate as the main carbon source (16, 17). Cell extracts of both H\(_2\)-CO\(_2\)- and formalate-grown \textit{M. tationis} cells showed CO-DH activities of 47.5 and 15.8 nmol/min per mg of protein, respectively. In cell extracts of H\(_2\)-CO\(_2\)-grown \textit{M. thermophilicum} cells an activity of 20.5 nmol/min per mg of protein was measured. Cell extracts of the autotroph \textit{Methanobacterium thermoautotrophicum} showed a much higher CO-DH activity, 189 nmol/min per mg of protein. Bott et al. (1) reported that most hydrogenotrophic methanogens which depend on external acetate for growth contain no CO-DH activity. \textit{Methanospirillum hungatii} was the only heterotrophic methanogen tested (1) which could reduce CO\(_2\) to carbon monoxide by using CO-DH, but it lacked the ability to incorporate CO in acetyl-coenzyme A. The results presented above indicate that \textit{M. tationis} and \textit{M. thermophilicum} are facultative heterotrophic methanogens, with a CO-DH activity insufficient for optimal cell growth. Therefore, external acetate is needed.

Our data strongly support a similar pathway of CO\(_2\) reduction to methane in \textit{M. tationis} and \textit{M. thermophilicum} with respect to other methanogens. The novel tatioppterin has the same function as methanopterin and sarcinapterin. Obviously, the proton at the 7-position of the pterin chromophore does not change the biological activity of the pterin molecule in the reactions described above.

![FIG. 1. Structure of tatioppterin. This pterin differs from sarcinapterin in having an additional aspartate in the side chain of the molecule and in not having the 7-methyl group in the pterin moiety.](http://jb.asm.org/Downloaded from December 15, 2017 by guest)

| TABLE 1. Methane production by cell extracts of \textit{M. tationis} and \textit{M. thermophilicum} with different C1 substrates |
|-----------------|-----------------|-----------------|-----------------|
| C1 substrate\(^a\) | Specific methane-producing activity (nmol/min per mg of protein) of: | \textit{M. tationis} | \textit{M. thermophilicum} |
| H\(_2\)-CO\(_2\) | 0.38 | 0.56 |
| HCHO-H\(_2\) | 11.37 | 11.81 |
| HCHO-N\(_2\) | 5.32 | 7.45 |
| CH\(_3\)S-CoM-H\(_2\) | 7.73 | 8.91 |
| CH\(_3\)S-CoM-H\(_2\)-CO\(_2\) | 7.91 | 13.35 |

\(^a\) CoM, Coenzyme M.

\(^\text{Notes}\)}
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**LITERATURE CITED**


