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Methanogenesis Involving a Novel Carrier of C_1 Compounds in *Methanogenium tationis*

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The pathway of CO_2 reduction to methane in *Methanogenium tationis* and *Methanogenium thermophilicum* is similar to that observed in other methanogens. In *M. tationis* a novel pterin, tatiopterin, is present. This pterin appears to be a structural and functional analog of methanopterin and sarcinapterin. Folate could not substitute for tatiopterin.

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 *Methanogenium tationis* and *Methanogenium thermophilicum* neither methanopterin nor sarcinapterin was present (4). A novel pterin has been found in *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 *M. tationis* and *M. thermophilicum* is similar to that in other methanogens and that the novel pterin, tatiopterin, from *M. tationis* has the same function as methanopterin and sarcinapterin.

M. thermophilicum (DSM 2373) and *M. tationis* (DSM 2702) were cultured in a medium described by Zabel et al. (16, 17) on H_2-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). *Methanobacterium thermoautotrophicum* ΔH (DSM 1053) was cultured on H_2-CO_2 by the method of Schönheit et al. (14). Cells were harvested by continuous centrifugation and stored at −70°C under an N_2 atmosphere.

Cell extracts from *Methanobacterium thermoautotrophicum* ΔH were prepared as described before (11). Cell extracts from *M. tationis* and *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 × *g*. The supernatant was stored at −70°C under an 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 *M. tationis*, *M. thermophilicum*, and *Methanobacterium thermoautotrophicum*, respectively.

Cell extracts from *M. tationis* and *M. thermophilicum* were tested for their ability to produce methane from different C_1 substrates. The standard mixture for this assay contained 12 μmol of TES buffer (pH 7.0), 0.5 μmol of ATP, 5 μmol of $MgCl_2$, and 75 μl of cell extract (2 mg of protein) in a total volume of 200 μl. Conversion of CO_2 as a C_1 substrate was measured under an H_2-CO_2 atmosphere (80:20, vol/vol). Conversion of formaldehyde was measured by adding 0.1 μmol of formaldehyde to the standard mixture under an N_2 or H_2 atmosphere. Conversion of methylcoenzyme M was measured by adding 0.1 μmol of methylcoenzyme M to the standard mixture under an H_2 or H_2-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 *Methanobacterium thermoautotrophicum* and the cofactors of the *Methanogenium* species, cofactor-free cell extract from *Methanobacterium thermoautotrophicum* was tested for the ability to produce methane from formaldehyde as a C_1 substrate under an H_2 atmosphere in a standard assay mixture supplemented with 50 μl of BCE from *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 μM, and methane production was measured.

Methylenetetrahydromethanopterin dehydrogenase (methylene-H_4MPT-DH) was measured, as described before (2), by monitoring the conversion of tetrahydromethanopterin (H_4MPT) to methenyl-H_4MPT in a mixture containing 180 μmol of potassium phosphate buffer (pH 6.0), 50 μmol of H_4MPT, 86 μmol of coenzyme F_{420}, 10 μmol of formaldehyde, and 20 μl of cell extract (5 μg of protein) in a final volume of 2 ml.

Methylene-H_4MPT reductase (methylene-H_4MPT-RD) activity was measured by monitoring the formation of methenyl-H_4MPT from methyl-H_4MPT in a mixture as described above for the methylene-H_4MPT-DH assay, except that 50 μg of protein was used and the reaction was started by adding 50 μmol of methyl-H_4MPT instead of H_4MPT. The formation of methenyl-H_4MPT and the reduction of coenzyme F_{420} 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 methylene-H_4MPT-DH and methylene-H_4MPT-RD activities, with tetrahydrofolate derivatives instead of H_4MPT derivatives. Aside from

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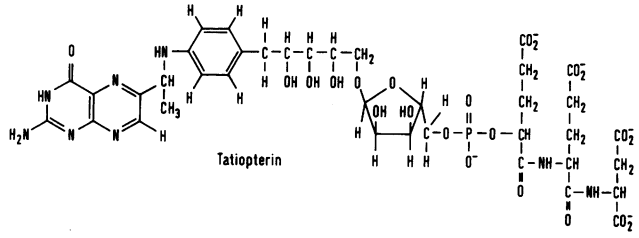

FIG. 1. Structure of tatiopterin. This pterin differs from sarcinapterin in having an additional aspartate in the side chain of the molecule and in not having the 7-methyl group in the pterin moiety.

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 $Na_2S_2O_4$ was replaced by 2 mM dithiothreitol.

Recently we reported the presence of a novel pterin in *M. tationis* (Raemakers-Franken et al., in press). Here we report cross-reactivity between enzymes of *Methanobacterium thermoautotrophicum* and pterins of *Methanogenium* species. Cofactor-free extract from *Methanobacterium thermoautotrophicum* was shown to convert formaldehyde to methane if BCE from *Methanogenium* species was added, with an efficiency of about 20% with respect to addition of BCE from *Methanobacterium thermoautotrophicum*. Thus, the tatiopterin derivatives present in BCE from *Methanogenium* species are recognized by the enzymes of *Methanobacterium thermoautotrophicum*. The occurrence of cross-reactivity was further demonstrated by our observation that methylene-H_4MPT-DH activity could be measured in cell extracts of *M. tationis* and *M. thermophilicum* (8.0 and 7.1 μmol/min per mg of protein, respectively) if H_4MPT (isolated from *Methanobacterium thermoautotrophicum*) was added. If H_4MPT was omitted, to check for an internal C_1 carrier, no reaction (activity lower than 0.2 μmol/min per mg of protein) was measured. Likewise, methylene-H_4MPT-RD activity could be measured in cell extracts of *M. tationis* and *M. thermophilicum* (123 and 76 nmol/min per mg of protein, respectively) if methyl-H_4MPT isolated from *Methanobacterium thermoautotrophicum* was added. Thus, the enzyme systems of *M. tationis* and *M. thermophilicum* are able to recognize and interconvert H_4MPT derivatives from *Methanobacterium thermoautotrophicum*. These results indicate that tatiopterin 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 C_1 carriers and NADP as the electron carrier. Previous work showed that H_4MPT cannot be replaced by tetrahydrofolate in the methylene-H_4MPT-DH reaction in *Methanobacterium thermoautotrophicum* (3, 6). Since the chromophore of tatiopterin contains a proton at the 7-position, just as folate derivatives do, we tested whether tetrahydrofolate could substitute for tetrahydrotatiopterin in cell extracts from *M. tationis* and *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 methyltetrahydrofolate was added to cell extracts from *Methanogenium* species in combination with coenzyme F_{420} or NADP as the electron acceptor. In this assay, activities lower than 0.12 μmol/min per mg of protein could not be measured. These results indicate that tetrahydrotatiopterin is functionally more related to H_4MPT and tetrahydrosarcinapterin than to tetrahydrofolate.

Cell extracts from *M. tationis* and *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 μM), 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 RPG 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 *M. tationis* and *M. thermophilicum* are able to grow on formate, the presence of formate dehydrogenase activity has to be expected. Extracts prepared from *M. tationis* cells grown on either H_2-CO_2 or formate appeared to contain this enzyme. Activities of 8.9 and 55.1 μmol/min per mg of protein, respectively, were measured. In the cell extract of *M. thermophilicum*, grown on H_2-CO_2, an activity of 17.9 μmol/min per mg of protein could be measured.

M. tationis and *M. thermophilicum* are heterotrophs, which grow on H_2-CO_2 or formate as an energy source but require acetate as the main carbon source (16, 17). Cell extracts of both H_2-CO_2- and formate-grown *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 *M. thermophilicum* cells an activity of 20.5 nmol/min per mg of protein was measured. Cell extracts of the autotroph *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. *Methanospirillum hungatei* 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 *M. tationis* and *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 *M. tationis* and *M. thermophilicum* with respect to other methanogens. The novel tatiopterin 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.

TABLE 1. Methane production by cell extracts of *M. tationis* and *M. thermophilicum* with different C_1 substrates

C_1 substrate[a]	Specific methane-producing activity (nmol/min per mg of protein) of:	
	M. tationis	*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_3S–CoM–H_2	7.73	8.91
CH_3S–CoM–H_2-CO_2	7.91	13.35

[a] CoM, Coenzyme M.

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