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A reversed genetic approach reveals the coenzyme specificity and other catalytic properties of three enzymes putatively involved in anaerobic oxidation of methane with sulfate

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Summary

Consortia of anaerobic methanotrophic (ANME) archaea and delta-proteobacteria anaerobically oxidize methane coupled to sulfate reduction to sulfide. The metagenome of ANME-1 archaea contains genes homologous to genes otherwise only found in methanogenic archaea, and transcription of some of these genes in ANME-1 cells has been shown. We now have heterologously expressed three of these genes in *Escherichia coli*, namely those homologous to genes for formylmethanofuran : tetrahydromethanopterin formyltransferase, methenyltetrahydromethanopterin cyclohydrolase (Mch) and coenzyme F_{420}-dependent methylenetetrahydromethanopterin dehydrogenase (Mtd), and have characterized the overproduced enzymes with respect to their coenzyme specificity and other catalytic properties. The three enzymes from ANME-1 were found to catalyse the same reactions and with similar specific activities using identical coenzymes as the respective enzymes in methanogenic archaea, the apparent K_m for their substrates being in the same concentration range. The results support the proposal that anaerobic oxidation of methane to CO_2 in ANME involves the same enzymes and coenzymes as CO_2 reduction to methane in methanogenic archaea. Interestingly, the activity of Mch and the stability of Mtd from ANME-1 were found to be dependent on the presence of 0.5–1.0 M potassium phosphate, which suggested that ANME-1 archaea contain high concentrations of lyotropic salts, presumably as compatible solutes.

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Introduction

Consortia of anaerobic methanotrophic (ANME) archaea and delta-proteobacteria mediate anaerobic oxidation of methane (AOM) coupled to sulfate reduction (Orphan *et al.*, 2001; Hallam *et al.*, 2004; Reeburgh, 2007). ANME archaea are phylogenetically classified into three groups that are all closely related to methanogenic archaea (Losekann *et al.*, 2007; Knittel and Boetius, 2009; Meyerdierks *et al.*, 2010). The taxonomic position of ANME-1 archaea is not well resolved, although it has been suggested in some reports that they are affiliated with Methanomicrobiales. ANME-2 and ANME-3 archaea are affiliated with Methanosarcinales.

Many methanogenic archaea produce methane from CO_2 and H_2 (Fig. 1), and gain energy from this process (Shima *et al.*, 2002; Thauer *et al.*, 2008; Thauer, 2011). The central methanogenic pathway, i.e. reactions from CO_2 reduction to methane production, are common to most of methanogens. In methanogenesis from the C1 compounds; methanol, methylamine and methylthiol carried out by Methanosarcinales, a portion of the C1 compound methyl group is oxidized via reversal of part of the central methanogenic pathway (Thauer, 2012). The metagenome of ANME-1 archaea contains genes homologous to genes encoding all enzymes in the central methanogenic pathway except the gene for methylenetetrahydromethanopterin reductase (Mer) (Meyerdierks *et al.*, 2010). The metagenome of an ANME-2d archaeon that grows by anaerobic methane oxidation coupled to nitrate reduction contains genes homologous to all known methanogenic genes, including the gene encoding Mer (Haroon *et al.*, 2013).

Based on the phylogenetic position of ANME archaea, and biochemical and metagenomic analyses, it has been proposed that they oxidize methane via reversal of the methanogenic pathway (Hallam *et al.*, 2004; Thauer, 2011). In this hypothesis, electrons obtained by methane

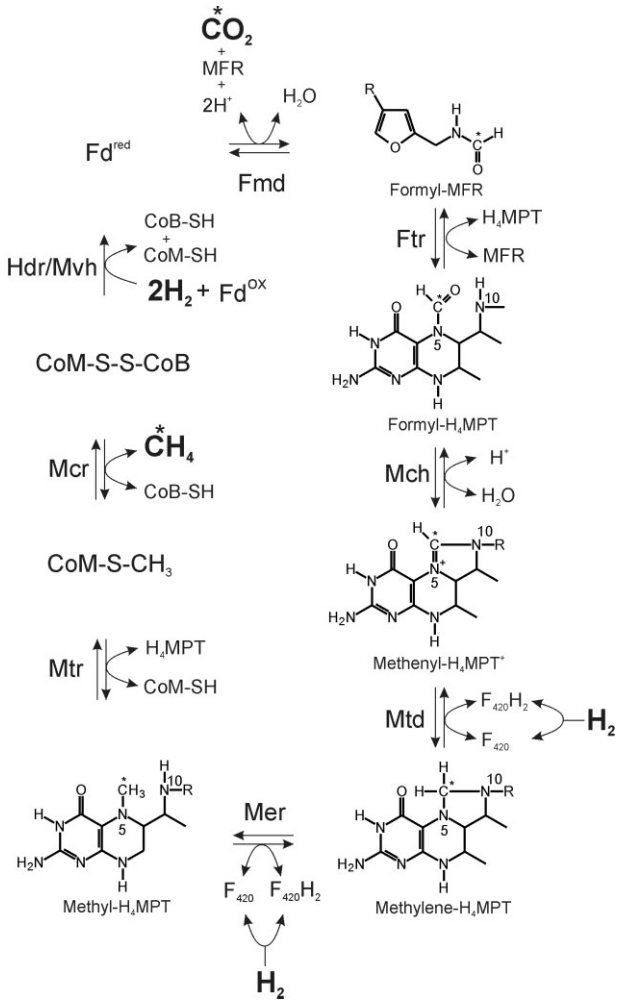

Fig. 1. Hydrogenotrophic methanogenic pathway of methanogenic archaea without cytochromes. Fmd, formylmethanofuran dehydrogenase; Mtr, integral membrane methyltransferase, whose reaction is coupled to sodium-ion extrusion; Hdr/Mvh, complex of heterodisulfide reductase and F_{420}-non-reducing hydrogenase. The reduced form of F_{420} is regenerated by F_{420}-reducing hydrogenase (Frh). Coenzyme M (CoM-SH) and coenzyme B (CoB-SH) are regenerated by Hdr/Mvh with the electron from H_2. Heterodisulfide reductase catalyses electron bifurcation reactions, by which four electrons from two H_2 are used for reduction of a heterodisulfide and a ferredoxin. Reduced ferredoxin (Fd^{red}) is supplied for the first reaction of the methanogenic pathway catalysed by Fmd and is also regenerated by the reaction catalysed by the integral-membrane energy-conserving hydrogenase, in which the endergonic reaction is coupled with sodium-ion influx into the cytoplasm. Reversible enzyme reactions are indicated with reaction arrows in both directions. All reactions other than Hdr are known to catalyse reverse reactions. Mer is not found in the ANME-1 metagenome.

oxidation are transferred to the associating bacteria, although the intermediates (electron carriers) for the syntrophy are not fully known. Recently, Milucka and colleagues (2012) proposed that ANME-2 archaea anaerobically oxidize methane with sulfate alone without associated bacteria. Sulfate was shown to be reduced to zero-valent sulfur in ANME-2 and the zero-valent sulfur to diffuse from the archaeal cells to the sulfate reducing bacteria, in which the zero-valent sulfur is disproportionated to sulfate and sulfide in a growth supporting exergonic reaction.

To test the hypothesis of reverse methanogenesis in ANME archaea, the respective enzymes must be characterized. However, such enzymological studies are not straightforward as ANME archaea have not yet been obtained in pure culture. Although enrichments of ANME-1 archaea that could be used for enzymological studies are available, purification of enzymes from enrichment cultures can be very difficult because of the limited availability of biomass because of low growth rates. Of the enzymes proposed to be involved in reverse methanogenesis, only the methyl-coenzyme M reductase (Mcr) homologue has been purified from a Black Sea microbial mat containing ANME-1 archaea mediating AOM (Krüger *et al*., 2003). This enzyme occurs in large amounts in the mat and was purified by ammonium sulfate fractionation and anion-exchange chromatography. The ANME-1 mat extract contained substances that interfered with chromatography. These substances were precipitated by 3 M ammonium sulfate (Krüger *et al*., 2003). However, ammonium sulfate precipitates not only unknown substances but also a lot of proteins in the microbial mat, which cannot then be separated from the unknown substances. The ANME-1 Mcr homologue has been crystallized (Shima *et al*., 2012). Scheller and colleagues (2010) have reported that Mcr isolated from the methanogenic archaeon *Methanothermobacter marburgensis* can catalyse methane oxidation at a rate that would be sufficient for supporting the AOM reaction mediated by the Black Sea microbial mat. The catalytic properties of other enzymes homologous to methanogenic enzymes encoded in the ANME-1 metagenome are still elusive.

The methanogenic pathway uses not only unique enzymes but also uncommon coenzymes, most of which are found only in methanogenic archaea and sulfate-reducing archaea (Dimarco *et al*., 1990). Although methanogenic coenzymes can be detected chemically, spectroscopically or mass spectrometrically, it is difficult to prove their presence in ANME archaea because these organisms coexist with methanogenic archaea in microbial ecosystem mediating AOM (Hinrichs *et al*., 1999; Tourova *et al*., 2002). The first evidence for the presence of methanogenic coenzymes in ANME-1 archaea was the binding of coenzyme M and coenzyme B to the active site of Mcr prepared from the Black Sea mat observed in the protein crystal structure (Shima *et al*., 2012). No evidence for methanofuran (MFR), tetrahydromethanopterin (H_4MPT) and F_{420} in ANME archaea has been obtained to date.

Because many methanogenic enzymes have metal-containing prosthetic groups or are post-translationally modified or both (e.g. methylation of amino acid side chains and thioglycine in Mcr and non-prolyl-*cis*-peptide bond in Mer) (Shima *et al*., 2002), heterologous production of these enzymes is difficult. Enzymes suitable for heterologous studies are those composed of only protein that is not post-translationally modified, i.e. formyl-MFR : H_4MPT formyltransferase (Ftr), methenyl-H_4MPT cyclohydrolase (Mch) and F_{420}-dependent methylene-H_4MPT dehydrogenase (Mtd) (Shima *et al*., 1995; Grabarse *et al*., 1999; Hagemeier *et al*., 2003). Here, we report the heterologous production of Ftr, Mch and Mtd homologues encoded by genes synthesized based on the ANME-1 archaeal metagenome protein sequences (Supporting Information Fig. S1). The produced enzymes utilized MFR, formyl-MFR, H_4MPT, formyl-H_4MPT, methenyl-H_4MPT, methylene-H_4MPT, F_{420} and a reduced form of F_{420} as substrates or products. The properties of the purified enzymes indicated that they catalyse the respective reactions in ANME-1 archaea using methanogenic coenzymes.

Results and discussion

Cell extracts of recombinant *Escherichia coli* strains that produced Ftr, Mch or Mtd homologues from synthesized genes based on the metagenome sequences of ANME-1 exhibited the respective enzyme activity. Because some methanogenic genes directly cloned from methanogenic archaea are very difficult to express in *E. coli*, codon usage of the genes and mRNA structure were optimized for *E. coli* expression. SDS-PAGE of cell extracts indicated that the apparent molecular mass of each heterologously overproduced protein is identical to that deduced from the genome sequence (Supporting Information Fig. S2). Although gene expression was performed at 20°C, large proportions of the enzymes formed inclusion bodies in which the enzymes were incorrectly folded and inactive. We purified soluble, active enzymes from cell extracts without affinity tags to avoid alteration of enzyme properties. SDS-PAGE of each purified enzyme fractions indicated that the enzymes were highly purified (Supporting Information Fig. S2). To confirm the sequence of the proteins purified, the respective protein bands of heterologously overproduced protein in cell extracts and in purified fractions were extracted from the SDS-polyacrylamide gel, digested by trypsin and sequenced using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The MALDI-TOF-MS sequences indicated that the respective gene products were overproduced and purified (Supporting Information Fig. S3).

Purification and properties of the Ftr homologue

The Ftr homologue was overproduced in *E. coli*. More than 90% of the heterologously produced enzyme was precipitated by centrifugation at $5000 \times g$ for 15 min at 4°C. This finding indicated that large proportions of the Ftr homologue formed inclusion bodies. Active Ftr in the supernatant of 1.6 M ammonium sulfate fractions was purified with Phenyl-Sepharose and Q-Sepharose chromatography (for the SDS-PAGE result, see Supporting Information Fig. S2). We assayed enzyme activity by measuring the increase in absorbance at 282 nm (formation of formyl-H_4MPT from H_4MPT and formyl-MFR), which is in the forward direction with respect to methanogenesis. Assay of the reverse reaction (direction in anaerobic methane oxidation) is difficult to measure because the free-energy change of the reverse direction is close to equilibrium but endergonic ($\Delta G^{\circ\prime} = +4.4$ kJ mol^{-1}); therefore, the reverse reaction stopped almost as soon as a small amount of substrate was converted to product. The formation of formyl-H_4MPT from H_4MPT was confirmed by the change in the UV-Vis spectrum (Fig. 2A); the major peak at ~300 nm shifted to ~280 nm. The shift was identical to that observed in the reaction of Ftr purified from methanogenic archaea (Breitung and Thauer, 1990). The specific activity of the purified fraction at 20°C was 460 U mg^{-1} (290 U mg^{-1} at 10°C) (Supporting Information Fig. S4). The specific activity was almost identical in the assay buffer containing 0.5 M potassium phosphate, which indicated that the enzyme is not salt-dependent. To check whether N^5-formyl-H_4MPT$^+$ was formed by the reaction catalysed by the Ftr homologue, we added Mch purified from *Methanothermococcus thermolithotrophicus* to the Ftr reaction mixture. Because the Mch cyclization reaction is favourable at acidic pH, the coupled assay was performed at pH 6.0 (the standard assay buffer for Ftr). In the Ftr/Mch coupled reaction, the absorbance at 336 nm stoichiometrically increased, which revealed formation of N^5,N^{10}-methenyl-H_4MPT$^+$ from N^5-formyl-H_4MPT. This finding indicated that N^5-formyl-H_4MPT rather than N^{10}-formyl-H_4MPT was produced by the reaction catalysed by Ftr from ANME-1 archaea. The kinetic properties of Ftr were analysed using the Lineweaver–Burk plot of the purified enzyme at 10°C (Fig. 2B). The lines in Fig. 2B were nearly parallel, as has also been observed with Ftr purified from *Methanopyrus kandleri*, which catalyses the reaction with a ternary complex mechanism (Breitung *et al*., 1992). The K_m and V_{max} values for H_4MPT were 80 μM and 900 U mg^{-1} respectively (Fig. 2C). The apparent K_m value of formyl-MFR was approximately 10 μM. Curve-fitting data were in agreement with those from Lineweaver–Burk plots (Supporting Information Fig. S5). The V_{max} of the Ftr homologue from ANME-1 archaea was

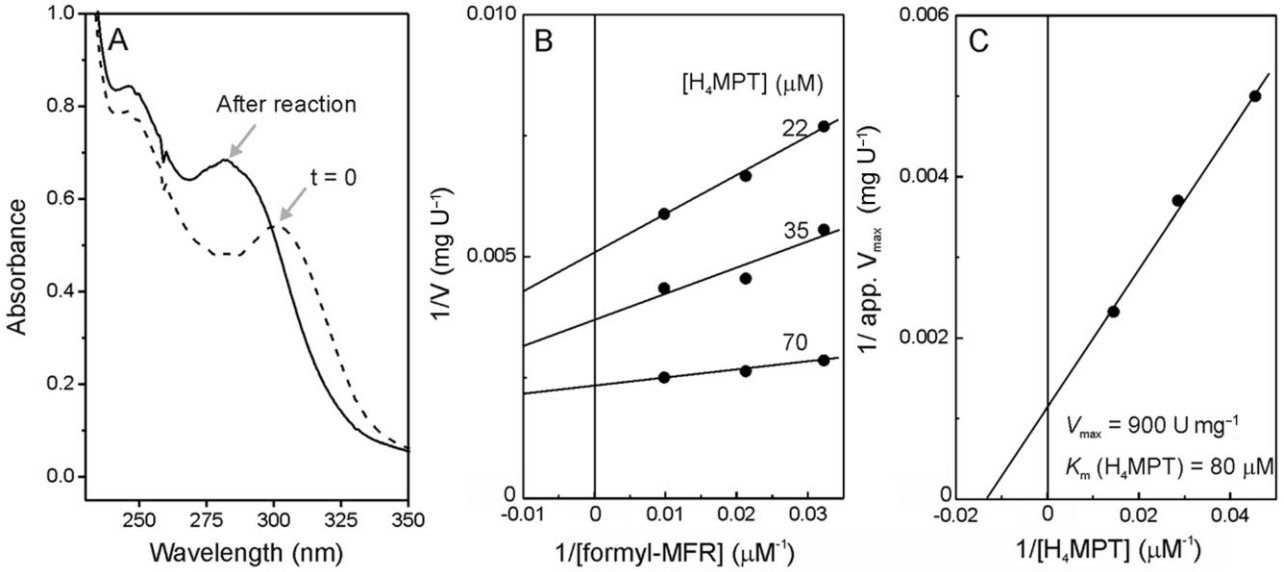

Fig. 2. Enzyme activity assays of the homolog of formylmethanofuran : H_4MPT formyltransferase (Ftr). (A) UV-Vis spectrum at 10°C of the substrates before addition of the enzyme (t = 0) and of the products after the enzyme reaction (after reaction). The assays were performed at 10°C in 0.7 ml total volume containing 120 mM potassium phosphate pH 6.0 and substrates at the concentration indicated. The reaction was started by adding 0.1 μg purified enzyme (final concentration in the assay was 4 nM). (B) Double-reciprocal plot of activity versus formyl-MFR concentration at different fixed concentrations of H_4MPT at 10°C. (C) Double-reciprocal plot of apparent V_{max} versus the reciprocal of the H_4MPT. Data were taken from (B).

lower than Ftr from the organisms listed in Table 1, and its K_m value was similar or even lower than those given for various organisms in Table 1 (Breitung and Thauer, 1990; Breitung *et al.*, 1992; Schwörer *et al.*, 1993). When tetrahydrofolate (functionally and structurally similar to H_4MPT) was used instead of H_4MPT, the specific activity was less than 1 U mg^{-1}.

Purification and properties of Mch

We assayed Mch activity by measuring the decrease in absorbance at 336 nm (formation of formyl-H_4MPT from methenyl-H_4MPT^+) at pH 7.6. This reaction is in the reverse direction with respect to methanogenesis, as expected in ANME archaea. Assay of the other direction is possible under acidic conditions. But we measured only one direction because of unavailability of purified formyl-H_4MPT as a substrate. Mch activity in cell extract was dependent on the presence of high concentrations of potassium phosphate. The activity in 1.0 M potassium phosphate buffer pH 7.6 was eightfold higher than that in 0.12 M potassium phosphate buffer pH 7.6. Therefore, we assayed Mch activity in 1.0 M potassium phosphate.

The heterologously produced Mch homologue was soluble in the absence of ammonium sulfate at pH 7.0. However, more than 90% of the Mch homologue was

Table 1. Properties of the homolog of Ftr from ANME-1, *Methanosarcina barkeri*, *Archaeoglobus fulgidus* and *Methanopyrus kandleri*.

Organism	Calculated mass of subunit (Da)	Calculated isoelectric point	Amino acid sequence identity (%)	Hydrophobicity	Optimum temperature for activity (°C)	V_{max} (U mg^{-1}) (Assay temperature)	K_m (μM)
ANME-1	31 864	4.7	100	+27.6	40	900 (10°C)	~10 (fMFR) 80 (H_4MPT)
Methanosarcina barkeri[a]	31 701	4.9	66	+21.6	65	3700 (37°C)	400 (fMFR) 400 (H_4MPT)
Archaeoglobus fulgidus[b]	31 761	5.0	58	+10.9	70	3300[d] (65°C)	32[d] (fMFR) 17[d] (H_4MPT)
Methanopyrus kandleri[c]	31 664	4.2	58	–31.4	90	2700 (65°C)	50 (fMFR) 100 (H_4MPT)

a. Breitung and Thauer 1990.
b. Schwörer *et al.*, 1993.
c. Breitung *et al.*, 1992.
d. Apparent V_{max}/K_m.

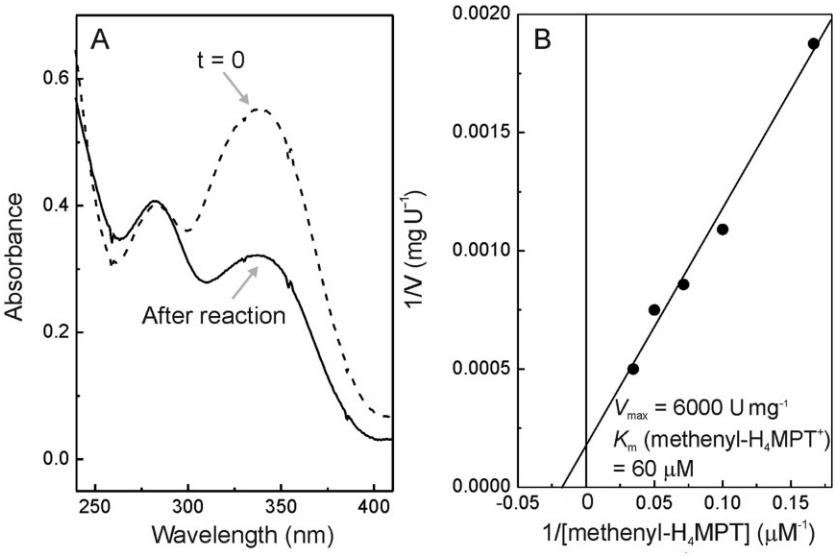

Fig. 3. Enzyme activity assays of the homologue of Mch. (A) UV-Vis spectrum at 10°C of the substrates before addition of the enzyme (t = 0) and of the products after the enzyme reaction (after reaction). The assay contained 1.0 M potassium phosphate pH 7.6 and methenyl-H_4MPT^+ as indicated. The reaction was started by adding 0.05 μg purified Mch (final concentration in the assay was 2 nM). (B) Lineweaver–Burk plot of Mch activity at 10°C dependent on the methenyl-H_4MPT^+ concentration. The 0.7 ml assay mixture contained 1 M potassium phosphate pH 7.6 and methenyl-H_4MPT^+ at the concentration indicated.

precipitated in the presence of 0.8 M ammonium sulfate at pH 7.0, and the precipitated protein was removed by centrifugation. The sedimented protein fraction contained only 1.5% of the total Mch activity; more than 80% of the Mch activity was found in the soluble fraction in the presence of 2.3 M ammonium sulfate at pH 7.0. This finding indicated that large proportions of the Mch homologue might be incorrectly folded and inactive. The Mch homologue in the supernatant of the 2.3 M ammonium sulfate fraction was purified using Phenyl-Sepharose and Q-Sepharose chromatography. This enzyme bound strongly to Phenyl-Sepharose and Q-Sepharose columns at pH 7.0, as has also been observed in the purification of Mch from *Methanosarcina barkeri* (Te Brommelstroet *et al.*, 1990). These characteristics were useful in isolating the tiny amount of active Mch homologue from endogenous *E. coli* proteins. SDS-PAGE indicated that the Mch homologue was the major protein in the final fraction (Supporting Information Fig. S2). The specific activity of purified enzyme was 3400 U mg^{-1} at 20°C (2000 U mg^{-1} at 10°C) (Supporting Information Fig. S4). This purification resulted in a 14 000-fold increase in the specific activity with an activity yield of 45%.

N^5,N^{10}-Methenyl-H_4MPT was hydrolysed by Mch as revealed by the UV-Vis spectrum change, in which the absorbance of the methenyl group of the substrate at 336 nm decreased as the Mch reaction proceeded (Fig. 3A). Lineweaver–Burk plot indicated V_{max} and K_m values of 6000 U mg^{-1} and 60 μM respectively (Fig. 3B). Curve-fitting data were in agreement with those from Lineweaver–Burk plot (Supporting Information Fig. S5). The V_{max} for ANME-1 Mch was slightly lower than that of the organisms listed in Table 2, but its K_m value was similar or even lower than those given for the various organisms in Table 2 (Te Brommelstroet *et al.*, 1990; Breitung *et al.*, 1991; Klein *et al.*, 1993b).

Purification and properties of Mtd

We assayed Mtd activity by measuring the decrease in absorbance at 401 nm (formation of reduced F_{420} from oxidized F_{420}) at pH 6.0. This reaction is in the reverse

Table 2. Properties of the homologue of Mch from ANME-1, *Methanosarcina barkeri*, *Archaeoglobus fulgidus* and *Methanopyrus kandleri*.

Organism	Calculated mass of subunit (Da)	Calculated isoelectric point	Amino acid sequence identity (%)	Hydrophobicity	Optimum temperature for activity (°C)	V_{max} (U mg^{-1}) (Assay temperature)	K_m (μM)
ANME-1	34,982	4.3	100	+11.0	50	6 000 (10°C)	60 (methenyl-H_4MPT^+)
Methanosarcina barkeri[a]	34,889	4.3	64	+11.0	50	8 000[d] (37°C)	570[d] (methenyl-H_4MPT^+)
Archaeoglobus fulgidus[b]	34,851	4.3	49	–7.7	85	11 300 (65°C)	220 (methenyl-H_4MPT^+)
Methanopyrus kandleri[c]	33,972	3.8	52	+1.8	95	13 300 (65°C)	40 (methenyl-H_4MPT^+)

a. Te Brommelstroet *et al.*, 1990.
b. Klein *et al.*, 1993a.
c. Breitung *et al.*, 1991.
d. Calculated from Te Brommelstroet *et al.*, 1990.

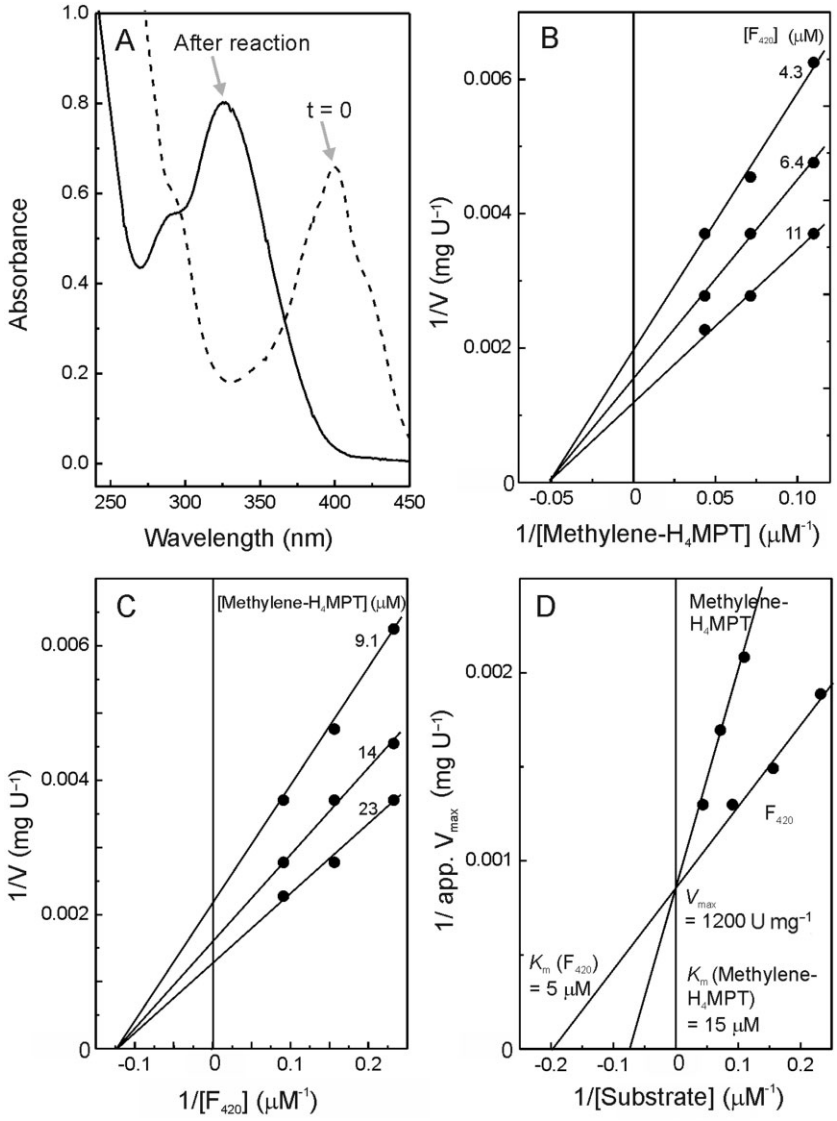

Fig. 4. Enzyme activity assays of the homologue of Mtd. (A) UV-Vis spectrum at 10°C of the substrates before addition of the enzyme (t = 0) and of the products after the enzyme reaction (after reaction). (B) Double-reciprocal plot of activity versus methylene-H_4MPT concentration at different fixed concentrations of F_{420}. The assays were performed at 10°C in a 0.7 ml total volume containing 0.5 M potassium phosphate pH 6.0 and substrates at the concentration indicated. The reaction was started by adding 0.02 μg purified Mtd (final concentration in the assay was 1 nM). (C) Double-reciprocal plot of activity versus F_{420} concentration at different fixed concentrations of methylene-H_4MPT. (D) Double-reciprocal plot of apparent V_{max} versus the reciprocal of the concentration of the substrates, F_{420} and methylene-H_4MPT, as indicated; data were taken from (A) and (B) respectively.

direction with respect to methanogenesis, as expected in ANME archaea. Assay of the other direction is theoretically possible under alkaline conditions. But we could not isolate reduced F_{420} as a substrate. Activity of the Mtd homologue in cell extract was very unstable when the enzyme was diluted in 120 mM potassium phosphate buffer pH 6.0 at lower temperature (< 10°C). The half-life of Mtd activity under these conditions on ice was less than 1 h. When cell extract was diluted in the same buffer and then incubated at room temperature, the inactivation rate was much lower (Supporting Information Fig. S6). This suggested that Mtd is cold-denatured (Privalov, 1990). Higher concentrations of potassium phosphate stabilized the enzyme at lower temperatures, but 1 M NaCl, 2 mg ml^{-1} BSA or 1 mM dithiothreitol did not. The optimal concentration of potassium phosphate for the Mtd activity stabilization was 0.5–0.8 M (Supporting Information Fig. S6). Lyotropic salts (i.e. potassium phosphate and other salts, which have strong salting-out effect on protein) are known to affects on stability of proteins (Breitung *et al.*, 1992).

Approximately half of the Mtd homologue was sedimented by ultracentrifugation at 120 000 × *g* for 30 min at 4°C, which indicated that the Mtd homologue formed inclusion bodies. The Mtd homologue in the supernatant of the 2.3 M ammonium sulfate fraction was purified using Phenyl-Sepharose and Q-Sepharose chromatography (for the SDS-PAGE result, see Supporting Information Fig. S2). The absorbance at 401 nm, which corresponds to the oxidized form of F_{420}, decreased during the reaction catalysed by Mtd. Concomitantly, the absorbance at 336 nm increased, which indicated formation of methenyl-H_4MPT^+ from methylene-H_4MPT (Fig. 4A); methenyl-H_4MPT^+ absorbs strongly at 336 nm,

Table 3. Properties of the homologue of Mtd from ANME-1, *Methanosarcina barkeri*, *Archaeoglobus fulgidus* and *Methanopyrus kandleri*.

Organism	Calculated mass of subunit (Da)	Calculated isoelectric point	Amino acid sequence identity (%)	Hydrophobicity	Optimum temperature for activity (°C)	V_{max} (U mg^{-1}) (Assay temperature)	K_m (μM)
ANME-1	30 287	6.1	100	−40.8	70	1200 (10°C)	15 (methylene-H_4MPT) 5 (F_{420})
Methanosarcina barkeri[a]	29 820	6.5	52	−6.0	>60	4000 (37°C)	6[d] (methylene-H_4MPT) 25[d] (F_{420})
Archaeoglobus fulgidus[b]	29 644	5.3	60	−10.1	70	5000[d] (65°C)	17 (methylene-H_4MPT) 13 (F_{420})
Methanopyrus kandleri[c]	31 383	4.3	45	−59.9	75	4000 (65°C)	80 (methylene-H_4MPT) 20 (F_{420})

a. Enßle *et al*., 1991.
b. Schwörer *et al*., 1993.
c. Klein *et al*., 1993b.
d. Apparent V_{max} or K_m.

whereas methylene-H_4MPT as well as oxidized and reduced F_{420} do not. The specific activity of the purified enzyme at 20°C was 1100 U mg^{-1} (760 U mg^{-1} at 10°C) (Supporting Information Fig. S4). In the Lineweaver–Burk plots, the three lines went through the same point on the x-axis, which indicated the ternary complex mechanism of this enzyme (Fig. 4B and C). The V_{max} was 1200 U mg^{-1}, and the K_m values for methylene-H_4MPT and F_{420} were 15 and 5 μM respectively (Fig. 4D). The curve-fitting data were in agreement with those from Lineweaver–Burk plots (Supporting Information Fig. S5). The V_{max} was slightly lower than that of the organisms listed in Table 3, but its K_m value was similar or even lower than those given for the various organisms in Table 3 (Enßle *et al*., 1991; Klein *et al*., 1993a; Schwörer *et al*., 1993). When methylenetetrahydrofolate (functionally and structurally similar to methylene-H_4MPT) was used instead of methylene-H_4MPT, the specific activity was less than 0.01 U mg^{-1}. When NAD or NADP (functionally similar to F_{420}) was used instead of F_{420}, the specific activity was less than 0.001 U mg^{-1}.

Effect of temperature

The temperature optimum of the Mch and Mtd homologues was 50 and 70°C, respectively (Fig. 5), similar to those of enzymes from the mesophilic methanogen *Methanosarcina barkeri* (growth temperature optimum of 37°C) (Breitung *et al*., 1991; Enßle *et al*., 1991). However, the temperature optimum of the Ftr homologue was 40°C (Fig. 5), which is lower than that of the counterpart from *Methanosarcina barkeri*, even though the primary structures are highly similar (66% identity; Table 1) (Breitung and Thauer, 1990). ANME-1 archaea have optimum AOM activity (4–16°C) (Nauhaus *et al*., 2002). It is worthwhile to note that typical psychrophilic enzymes from microorganisms that grow optimally at < 4°C have an optimal temperature of ~30°C (Feller and Gerday, 2003).

Specific activity required for observed AOM

Heterologously produced Ftr, Mch and Mtd homologues revealed high specific activity at 10°C. The reported AOM rate of ANME-1 archaea in the Black Sea microbial mat under laboratory conditions of 14 bar CH_4 at 12°C is 230 μmol day^{-1} g (dry sediment)$^{-1}$, which corresponds to 5 mmol day^{-1} g (dry cell mass)$^{-1}$ (Nauhaus *et al*., 2002). The specific activity of methane oxidation has been calculated to be 7 nmol mg (protein)$^{-1}$ min^{-1} (7 mU mg^{-1}), assuming 50% of the dry cell mass is protein (Scheller

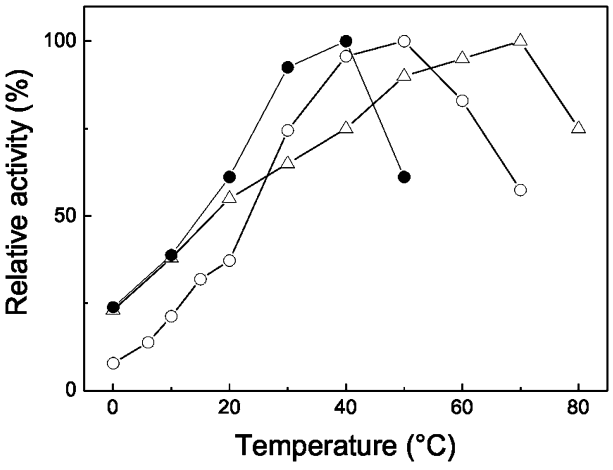

Fig. 5. Effect of temperature on the three methanogenic enzymes homologues. The activity was determined under standard assay conditions described in the Experimental procedure section. Filled circles, Ftr; open circles, Mch; open triangles, Mtd. Percentages of the optimum activity of each enzyme are plotted as relative activities.

et al., 2010). To date, protein concentration of the Ftr, Mch and Mtd homologues in ANME archaea were not determined, although expression of the *mtd* gene homologue was detected by mRNA analysis (Meyerdierks *et al*., 2010). Assuming that the content of each of the proteins investigated in ANME-1 archaea is 0.2–1%, as observed in methanogen cell extract (Breitung and Thauer, 1990; Te Brommelstroet *et al*., 1990; Enßle *et al*., 1991), the minimum specific activity of these purified enzymes required should be at least 100–500-fold higher than the observed methane-oxidation rate, which corresponds to 0.7–3.5 U mg^{-1}. The specific activity of each purified methanogenic enzyme at 10°C under standard conditions (Ftr, 290 U mg^{-1}; Mch, 2000 U mg^{-1}; and Mtd, 760 U mg^{-1}) was much higher (> 100 fold) than this estimated minimum value (0.7–3.5 U mg^{-1}), which indicated that Ftr, Mch and Mtd encoded in the metagenome can support the AOM reaction in ANME-1 archaeal cells.

Krüger and colleagues (2003) reported specific activities of these three methanogenic enzymes in cell extracts obtained from the Black Sea mat dominated by ANME-1 archaea, which contained 70% ANME-1 archaea, 25% sulfate-reducing bacteria and less than 5% other microorganisms. At 12°C, the specific activities of the cell extract were 20–100 mU mg^{-1} (Ftr), 200–700 mU mg^{-1} (Mch) and 20–50 mU mg^{-1} (Mtd). Assuming 1% of the cell mass of the mat is the coexisting methanogenic archaea, which exhibit enzyme activities as observed in *Methanosarcina barkeri*, the specific activities originating from the methanogens are estimated to be ~10 mU mg^{-1} (Ftr), ~100 mU mg^{-1} (Mch) and ~100 mU mg^{-1} (Mtd). However, Ftr and Mch activities detected in the Black Sea mat were substantially higher than these estimated values. The additional activity could reflect the activity produced in ANME-1 archaea. The measured Mch and Mtd activity could be underestimated because our study indicated that Mch activity and Mtd stability are dependent on lyotropic salt. Mtd from ANME-1 archaea in the mat extract appeared to be inactivated in the assay buffer, although the potential activity in the mat cell extract should be much higher than that observed.

Salt dependency of the enzymes

ANME-1 archaea form microbial mats on the floor of the Black Sea. To protect cells against osmotic pressure, most marine bacteria and archaea contain organic compounds rather than inorganic salts as compatible solutes. Some methanogens (e.g. *Methanothermobacter thermautotrophicus*, *Methanothermus fervidus*, *Methanopyrus kandleri*) contain a high concentration of cyclic diphosphoglycerate in the cytoplasm, and some methanogenic enzymes of these methanogens are dependent on lyotropic salts for activity or stability, or both (Shima *et al*., 1998; Roberts, 2005). Salt dependence of the activity and stability of the methanogenic enzymes from *Methanopyrus kandleri* have been extensively studied. The *Methanopyrus kandleri* enzymes can be stabilized and/or activated in the presence of high concentration of potassium phosphate or other lyotropic salt. Therefore, our finding of potassium phosphate dependency of ANME-1 enzymes suggested that ANME-1 archaeal cells could contain organic phosphate or other lyotropic organic compounds presumably as compatible solutes. In the methanogens of the Methanosarcinales and Methanomicrobiales closely related to ANME-1 archaea, betaine and Nε-acetyl-β-lysine are the most important compatible solutes (Roberts, 2005). Our findings suggest that in the study of enzyme activities of cell extracts of the Black Sea mat, enzyme assay buffers should contain high lyotropic salt concentrations in order to detect the activity.

Relevance to metagenome analysis

Metagenomic, metaproteomic and metatranscriptomic analyses are extensively used to explore the properties of unculturable microorganisms. These techniques are based on the potential function of genes that encode proteins similar to proteins (enzymes) of known functions. But to prove the functions of the proteins, the proteins have to be biochemically analysed, i.e. enzyme activities must be measured. This is particularly true when the predicted enzymes are expected to be involved in uncommon metabolisms or they use unusual coenzymes. A typical example of such a case is reverse methanogenesis in ANME archaea. In our study, we synthesized the genes encoding three enzymes involved in the hypothetical reverse methanogenesis based on metagenomic sequences, and heterologously produced, purified and characterized the enzymes. These Ftr, Mch and Mtd enzymes had an apparent high affinity to their specific coenzymes and reasonably high specific activity at the situ temperature. Our findings supported the hypothesis that these methanogenic enzymes of the ANME-1 archaea catalyse reverse methanogenic reactions using methanogenic coenzymes. Our results also indicated that such an approach of synthesizing genes based on metagenomic sequences could be used to elucidate the function of unculturable organisms in microbial communities.

Experimental procedures

The *ftr* (CBH38579), *mch* (CBH37751) and *mtd* (CBH37899) genes were chemically synthesized by GenScript (Piscataway, NJ, USA) based on the metagenome sequence (Meyerdierks *et al*., 2010); the codon usage was optimized

for expression in *E. coli*. (Supporting Information Fig. S1, sequences of synthesized genes.) The genes were cloned into expression vector pET24b and introduced into *E. coli* BL21(DE3) by transformation. MFR, H_4MPT, methenyl-H_4MPT and F_{420} were purified from *Methanothermobacter marburgensis*, and formyl-MFR and methylene-H_4MPT were chemically synthesized from MFR and H_4MPT as described previously (Shima and Thauer, 2001; Shima *et al*., 2011). NAD, NADP, and tetrahydrofolate were obtained from Sigma-Aldrich (Taufkirchen, Germany). The fast protein liquid chromatography (FPLC) columns were from GE Healthcare (Freiburg, Germany).

Heterologous expression

Recombinant *E. coli* BL21(DE3) strains harbouring the recombinant plasmids were cultivated in 2 l tryptone-phosphate medium at 37°C (Moore *et al*., 1993). When the cultures reached an optical density at 600 nm of ~1.0, they were cooled to 20°C, and 1 mM (final concentration) isopropyl-β-D-thiogalactopyranoside was added to induce gene expression. After 3 h of incubation at room temperature, the *E. coli* cells were harvested and frozen at −80°C. The *E. coli* strain without the recombinant vector did not show any activity of the methanogenic enzymes tested.

Protein purification

Frozen *E. coli* cells (6 g) were suspended in 14 ml of 50 mM Mops-KOH pH 7.0. The cells were disrupted with ultrasonication (SONOPULS GM200; Bandelin, Berlin, Germany) for 10 min (2 × 5 min) at 80 W (50 cycles). Cell debris and unbroken cells were removed by centrifugation at 5000 × *g* for 15 min at 4°C. The supernatant was subsequently ultracentrifuged at 120 000 × *g* for 30 min at 4°C.

The supernatants were fractionated with various concentrations of ammonium sulfate (see Results and discussion) on ice for 10 min, followed by centrifugation at 18 000 × *g* for 20 min at 4°C. The fractions containing the respective enzyme activity were pooled. Proteins were separated by column chromatography using the FPLC system (GE Healthcare, Freiburg, Germany) under air at room temperature. The ammonium sulfate concentration in the samples were adjusted to 1 M and then loaded onto a HiTrap Phenyl HP column (5 ml) equilibrated with Mops-KOH pH 7.0 containing 1 M ammonium sulfate. After washing with 25 ml of equilibration buffer, proteins were eluted with a linear decreasing gradient of ammonium sulfate (1 to 0 M) in 100 ml, followed by elution with 25 ml buffer without salt. Ftr eluted with pure water (5 ml), Mch eluted in the buffer without salt (10 ml) and Mtd eluted at ~0.9 M ammonium sulfate (40 ml). The buffer of the fractions containing each enzyme activity was exchanged with 50 mM Mops-KOH pH 7 using a 30 kDa cut-off ultrafilter. The samples were loaded onto a HiTrap Q HP column (5 ml) equilibrated with 50 mM Mops-KOH pH 7.0 (buffer). For purification of the Ftr homologue, the column was washed with 25 ml of 0.1 M NaCl in buffer, and then proteins were eluted in a 0.1–0.6 M NaCl gradient in buffer (100 ml); the Ftr homologue was recovered in two 5 ml fractions of ~0.35 M NaCl. For purification of the Mch homologue, proteins were eluted with a 0.1–0.6 M NaCl gradient in buffer (100 ml); the Mch homologue eluted in a 5 ml fraction at 1 M NaCl in buffer. For Mtd purification, the column was washed with 25 ml buffer, and proteins were eluted in a 0–0.6 M NaCl gradient in buffer (100 ml); the Mtd homologue was recovered in two 5 ml fractions at ~0.2 M NaCl. The purity of each 5 ml fraction was analysed by SDS-PAGE. The purest fraction of each protein based on SDS-PAGE analysis was used for enzyme characterization. Protein concentrations were assayed using the Coomassie Brilliant Blue G-250 dye-binding method with reagent from Bio-Rad (Munich, Germany). The increase in absorbance at 595 nm caused by binding of the dye to proteins in the assay solution was measured, and the protein concentration was calculated; BSA was used as the standard.

Activity assays

Enzyme activity of reactions catalysed in one direction was assayed as described previously in 1 ml quartz cuvettes (1 cm path length) with rubber stoppers under 100% N_2 atmosphere. The substrates were added anoxically to the cuvettes using Hamilton syringes. The enzyme reaction was started by adding enzyme solution (5–10 µl) to 0.7 ml assay buffer in the cuvette at the temperature indicated and then change of the absorbance indicated was recorded for at least ~1 min. One unit (U) activity is the amount of enzyme catalysing formation of 1 µmol of products per minute. For the standard assay of Ftr, 70 µM formyl-MFR and 43 µM H_4MPT (final concentrations in the cuvette) were added to 120 mM potassium phosphate pH 6.0. The formation of formyl-H_4MPT from H_4MPT and formyl-MFR was monitored by following the increase in absorbance at 282 nm ($\Delta\varepsilon = 5.1\ mM^{-1}\ cm^{-1}$) on a UV-Vis spectrophotometer (Ultrospec 1100; GE Healthcare) (Breitung *et al*., 1992). To check the formation of N^5-formyl- (rather than N^{10}-formyl-)H_4MPT, Mch purified from the methanogenic archaeon *Methanothermococcus thermolithotrophicus* (Hartmann *et al*., 1996) was added to the Ftr reaction mixture at pH 6.0. To determine the activity using other substrates, 43 µM tetrahydrofolate (final concentration) was added instead of H_4MPT; the reaction was started by adding1 µg purified Ftr homologue (final concentration in the assay was 0.04 µM). The change of spectra (200–600 nm region) was recorded with diode-array spectrophotometer (Specode S600, Analytik Jena, Jena, Germany) at 20°C for 10 min. For the standard assay of Mch, 30 µM methenyl-H_4MPT (final concentration) was added to 0.7 ml of 1 M potassium phosphate pH 7.6. The conversion of methenyl-H_4MPT$^+$ to formyl-H_4MPT was monitored by following the decrease in absorbance at 336 nm ($\Delta\varepsilon = 21.6\ mM^{-1}\ cm^{-1}$) (Breitung *et al*., 1991). For determining the optimal salt concentration, potassium phosphate or sodium chloride was added to the assay buffer. For the standard assay of Mtd, 25 µM F_{420} and 35 µM methylene-H_4MPT were added to 0.5 M potassium phosphate pH 6.0 at 20°C. The formation of the reduced form of F_{420} from its oxidized form was monitored by following the decrease in absorbance at 401 nm ($\Delta\varepsilon = 25.9\ mM^{-1}\ cm^{-1}$) (Enßle *et al*., 1991). To determine the activity using other substrates, 35 µM methylenetetrahydrofolate and 200 µM NAD(P) (final concentrations) were used instead of methylene-H_4MPT and F_{420} respectively. Methylenetetrahydrofolate was prepared from

tetrahydrofolate and 1.6 mM (final concentration) formaldehyde in an assay cuvette as described previously (Vorholt *et al.*, 2000). The reaction was started by adding 1.5 μg purified Mtd homologue (final Mtd concentration in the assay was 0.07 μM). For the assays of methylenetetrahydrofolate and F_{420}, and for the assay methylene-H_4MPT and NAD(P), the absorbance of 401 and 340 nm, respectively, was recorded at 20°C for 10 min. Activity of the purified enzymes was unstable and decreased during incubation in solution and when frozen. Therefore, the specific activity was determined immediately after purification. For time-consuming kinetics studies, only partially inactivated enzyme preparations were available; the original activities were calculated using the ratio between the original enzyme activity and the partially inactivated activity under standard assay conditions. The optimal temperature of the enzymes was determined using the same standard assay conditions except the assay temperature.

SDS-PAGE and MALDI-TOF-MS sequencing

Overexpression of the genes and purity of the purified enzymes were analysed by SDS-PAGE (Laemmli, 1970). Protein samples were dissolved in a sample buffer (50 mM Tris/HCl pH 6.8, 50 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol) and incubated for 5 min at 80°C. Protein was separated on 12% polyacrylamide gels (8 cm × 7 cm) and subsequently stained with Coomassie Brilliant blue G250 (PageBlue Protein Staining Solution, Thermo Scientific, Rockford, IL, USA).

Each SDS-polyacrylamide gel band was cut-out, chopped into small pieces, destained with 30% iso-propanol (v/v) containing 50 mM NH_4HCO_3, dehydrated with 100% iso-propanol and dried. Gel pieces were rehydrated in 5 mM NH_4HCO_3 in 10% acetonitrile (v/v) containing 2.5 mg l^{-1} sequencing-grade modified trypsin (Promega) and incubated for 10 h at 22°C. Tryptic peptides were extracted with 0.1% trifluoroacetic acid (v/v) in water. The extracted peptides were concentrated under vacuum but not to dryness. The resulting peptide mixture was injected onto a PepMap100 C-18 RP nanocolumn (Dionex, Idstein, Germany) equipped with UltiMate 3000 liquid chromatography system (Dionex) in a continuous acetonitrile gradient consisting of 0–40% B in 40 min, 40–100% B in 10 min [B: 80% (v/v) acetonitrile, 0.04% (v/v) trifluoroacetic acid] at a flow rate of 300 nl min^{-1}. A Probot microfraction collector (Dionex) was used to spot peptides separated by liquid-chromatography on a MALDI target at a rate of 29 s/spot. The fractions were mixed with a matrix composed of 3 mg ml^{-1} α-cyano-4-hydroxycinnamic acid in 80% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid. MALDI-TOF-TOF analysis was performed with a 4800 Proteomics Analyzer (Applied Biosystems/MDS Sciex, Forster City, CA, USA) using the 4800 Series Explorer software (positive-ion reflector mode in a mass range from 840 to 5000 Da with an S/N minimum set to 80). The data were calibrated externally using peptide mixture for mass calibration from Bruker (Bremen, Germany) spotted onto the same MALDI target. MS/MS data were searched against an in-house ANME-1 metagenome-database using Mascot embedded into GPS explorer software (MDS Sciex).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Synthesized nucleotide sequences and amino acid sequences of homologous genes for *ftr* (CBH38579), *mch* (CBH37751) and *mtd* (CBH37899) genes from the ANME-1 metagenome (Meyerdierks *et al*., 2010). The codon usages were optimized for expression in *Escherichia coli*. In the nucleotide sequences, the start and stop codons are in boldface.

Fig. S2. SDS-PAGE of purified enzymes. Lane 1, protein standards; lane 2, 10 μg cell extract containing the Ftr homologue; lane 3, 1.5 μg purified Ftr homologue; lane 4, 10 μg cell extract containing Mch homologue; lane 5, 1 μg purified Mch homologue; lane 6, 10 μg cell extract containing Mtd homologue; lane 7, 1.5 μg purified Mtd homologue. The gel was stained with Coomassie Brilliant Blue G250 (PageBlue Protein Staining Solution, Thermo Scientific, Rockford, IL, USA).

Fig. S3. Mass spectrometric sequencing of the overproduced enzymes in the cell extract and the purified enzymes. The corresponding bands in the SDS-PAGE in Supporting Information Fig. S2 were cut-out and analysed. (A) The preparations were degraded with trypsin, the peptides separated by nano-LC and the peptides analysed by MALDI-TOF-MS-MS and by the MASCOT search program against a database containing the sequences shown in the panel A. Amino acid sequences were obtained from ANME-1 metagenome. The sequence is identical to the ANME-1 enzymes sequence deduced from the DNA sequence of the gene synthesized. Mass spectroscopic analysis of the tryptic peptide revealed matching sequences that are highlighted in red. The results from the lane 2 (Ftr homologue), 4 (Mch homologue) and 6 (Mtd homologue) were shown. (B) The score is −10*Log (P), where *P* is the probability that the observed match is a random event.

Fig. S4. Representative raw data of the enzyme assay. Formyl-MFR:H_4MPT formyltransferase (Ftr), methenyl-H_4MPT cyclohydrolase (Mch) and F_{420}-dependent methylene-H_4MPT dehydrogenase (Mtd).

Fig. S5. Direct fitting of the mathematical relationship of the kinetic data using the Michaelis–Menten equation. The calculated kinetic parameters were compared with the data obtained from Lineweaver–Burk plots shown in Figures 2–4 in the main text (tables). The kinetic parameters obtained by two different methods were almost identical. Data of formylmethanofuran : tetrahydromethanopterin formyltransferase (Ftr), methenyltetrahydromethanopterin cyclohydrolase (Mch) and F_{420}-dependent methylenetetrahydromethanopterin dehydrogenase (Mtd) are presented.

Fig. S6. Effect of potassium phosphate concentrations on stability of the Mtd homologue. (A) Stability of the Mtd homologue in 120 mM potassium phosphate pH 6.0. Cell extract containing the Mtd homologue was diluted 5000-fold with 120 mM potassium phosphate pH 6.0 and incubated anoxically at 0°C (on ice), at 10°C and at 20°C. Twenty microlitres of the samples were withdrawn and assayed at 20°C. At lower temperatures, the Mtd activity decreased more rapidly. The inactivated enzyme was fully reactivated by incubation at 20°C for 1 h. (B) Salt-dependent stabilization of the Mtd homologue. Cell extract containing the Mtd homologue was diluted 400-fold with 0.12–1.0 M potassium phosphate buffer and incubated anoxically for 4.5 h. Ten microlitres of samples was withdrawn and assayed at 20°C. In the presence of 0.5–0.8 M potassium phosphate, no inactivation was observed. (C) Stability of purified Mtd homologue in 120 and 500 mM potassium phosphate concentrations at pH 6.0. The purified Mtd homologue was diluted to 1 μg ml^{-1} with potassium phosphate solutions and incubated anoxically on ice. The purified Mtd homologue was also inactivated on ice even in the presence of 500 mM potassium phosphate. The inactivation rate was higher in 120 mM potassium phosphate than in 500 mM potassium phosphate. The purified enzyme inactivated on ice was not reactivated by incubation at 20°C for 1 h.