Methanogenic conversion of 3-S-methylmercaptocaproionate* to 3-mercaptocaproionate

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*3-S-Methylmercaptocaproionate (MMPA) is the same compound as methylthiopropionate (MTPA) mentioned in the other chapters
Anaerobic metabolism of DMSP, an osmolyte of marine algae, in anoxic intertidal sediments involves either cleavage to dimethylsulfide or demethylation to methylmercaptocpropionate (MMPA) and subsequently to mercaptopropionate. The methanogenic archaea Methanosarcina sp. strain MTP4 (DSM 6636), Ms. acetivorans DSM 2834, and Ms. (Methanolobus) siciliae DSM 3028 were found to use MMPA as a growth substrate and to convert it stoichiometrically to mercaptopropionate. Approximately 0.75 mol methane was formed per mol MMPA degraded; methanethiol was not detected as an intermediate. Eight other methanogenic strains did not carry out this conversion. We also studied the conversion of MMPA in anoxic marine sediment slurries. Addition of MMPA (500 µM) resulted in the production of methanethiol which was subsequently converted to methane (417 µM). In the presence of the antibiotics ampicillin, vancomycin and kanamycin (20 µg/ml each), 275 µM methane was formed from 380 µM MMPA; no MT was formed during these incubations. Only methanethiol was formed from MMPA when 2-bromoethanesulfonate (25 mM) was added to a sediment suspension. These results indicate that in natural environments MMPA could be directly or indirectly a substrate for methanogenic archaea.

Introduction

DMSP, an osmolyte found in many marine algae and certain plants (Reed 1983; Keller 1988; Gröne and Kirst 1991), is the major source of dimethylsulfide in the marine environment. In the atmosphere dimethylsulfide contributes through its oxidation products methanesulfonic acid and sulfuric acid to cloud formation and acid precipitation (Charlson et al. 1987). DMSP and dimethylsulfide are biologically converted in oxic as well as anoxic environments (Kiene et al. 1986; Kiene 1988; Kiene and Capone 1988; Kiene and Taylor 1988a and b; Taylor and Gilchrist 1991). The production of DMSP by benthic algae and the settling of dead algae cause the presence of DMSP in marine sediments. Relatively high concentrations of DMSP (from approx. 1 up to 70-110 µmol/l sediment) have been measured in the surface sediment of a salt marsh and other types of intertidal sediments (Kiene 1988; Visscher et al. 1994). In estuarine carbonate sediments DMSP is degraded by cleavage to dimethylsulfide and acrylate or by a demethylation to MMPA and subsequently to mercaptopropionate (Kiene and Taylor 1988a and b). MMPA is also degraded to methanethiol and presumably acrylate (Kiene and Taylor 1988a); acrylic acid and hydrogen sulfide can slowly react to form mercaptopropionate in a chemical process (Variavamurthy and Mopper 1987). Kiene and Taylor (1988a) suggested that a Eubacterium limosum-like organism might be responsible for the sequential demethylation of DMSP to mercaptopropionate. Thus far, no anaerobic microorganisms have been isolated that are able to demethylate DMSP all the way to mercaptopropionate. Anoxic most probable number incubations (in the presence of 5 mM NO₃⁻) of mud from the upper 5 mm of a cyanobacterial mat indicated the presence of considerable populations of mercaptopropionate-producing microorganisms (Visscher et al. 1994), but the characteristics of the organisms are unknown. Recently, it was shown that the marine sulfate-reducing bacterium Desulfovibacterium strain PM4 demethylates DMSP to MMPA (van der Maarel et al. 1993). This organism is not able
Methanogenesis from MMPA to demethylate MMPA to mercaptopropionate. In this communication we present data that show that some methanogenic archaea are able to demethylate MMPA to mercaptopropionate.

Materials and methods

Sediment sampling, preparation, and incubation. Anoxic intertidal sediment was collected from the Wadden Sea near Westernieland, The Netherlands. The sediment consisted of a black sulfide-rich layer covered by a 0- to 2 cm-thick oxic sandy layer. Sediment cores were taken using perspex cores (10-cm length; 2.5-cm diameter). After sampling the cores were taken using perspex cores (10-cm length; 2.5-cm diameter). After sampling the cores were sealed with butylrubber stoppers. The samples were transported in a N₂-flushed anaerobic jar at ambient temperature and suspensions were made in an anaerobic glove box (equipped with catalyst R0-20 of BASF Aktiengesellschaft, Ludwigshafen, Germany) within a few hours. Fresh sediments contained 40-60 µM DMSP when measured as dimethylsulfide by headspace analysis after alkalinization of the sample with NaOH (final concentration, 5 M).

The sediment was suspended in degassed seawater (approximately 4 ml/g of sediment [wet weight]). The suspension was thoroughly mixed with a blender for one min and 40-ml aliquots were poured into 70-ml bottles, while the sediment was kept in suspension. The bottles were sealed with a screw cap containing a butyl rubber stopper through the central hole; a Viton disc which is impermeable to volatile sulfur compounds was placed beneath the rubber stopper (Dr. J. Gerritse, personal communication). The headspace was flushed with oxygen-free N₂ for 2 min. Then, if required, the inhibitor 2-bromoethanesulfonic acid (final concentration 25 mM) or the antibiotics ampicillin, vancomycin, and kanamycin (20 µg/ml final concentration each) were added from aqueous stocks and the suspension was incubated at 25°C overnight to remove remaining oxygen; then the incubations were started by addition of the substrate.

Microorganisms and growth conditions. An enrichment culture of MMPA-degrading microorganisms was obtained by inoculation of anoxic sediment (2.5-ml suspension) in bicarbonate-buffered (50 mM) mineral medium (Heijthuijsen and Hansen 1989) with sulfate (20 mM), yeast extract (50 mg/l), and MMPA (10 to 20 mM). Sulfate was omitted after several transfers into fresh medium. Incubations were done in 120-ml bottles filled with 50 ml medium at 28°C. *Methanosarcina* sp. strain MTP4 (DSM 6636) was grown in 120-ml bottles filled with 50 ml medium according to Finster et al. (1992) under an atmosphere of N₂-CO₂ (80/20) at 30°C; inoculation (5%) was from late-log phase cultures. Strain MTP4 was isolated with methanethiol as a substrate by Finster et al. (1992) from sediment of salt marsh near Bordeaux, France. Growth was followed by measuring the optical density at 430 nm. The following strains were also used: *Ms. acetivorans* MS (DSM 2834) precultured on methanol (10 mM); *Ms. mazeii* C16 (DSM 3318; also known as ‘*Ms. frisia*’ C16) precultured on methanol (25 mM); *Ms. siciliae* T4/M (DSM 3028; this strain was formerly designated *Methanolobus siciliae*; cf. Ni et al. 1994) precultured on methanol (25 mM); *Methanobacterium* sp. strain C8 (DSM 3821) precultured on H₂-CO₂ (80/20); *Methanococcoides methylutens* TMA-10 (DSM 2657) precultured on
trimethylamine (25 mM); *Methanohalophilus zhilinaeae* WeN5 (DSM 4017) precultured on trimethylamine (10 mM); *Methanospirillum hungateii* JF1 (DSM 864) precultured on H$_2$-CO$_2$ (80/20) and acetate (2.5 mM); *Ms. barkeri* Fusaro precultured on acetate (5 mM), and *Ms. barkeri* MS precultured on methanol (10 mM). *Ms. barkeri* strains Fusaro and MS were kindly provided by J.T. Keltjens, University of Nijmegen, The Netherlands. All of these strains were cultivated in the media as described in the Deutsche Sammlung von Mikroorganismen und Zellkulturen catalog of strains (1993, Braunschweig, Germany). *Methanococcoides* sp. strain PM2 (Culture collection of the Department of Microbiology, University of Groningen), precultured on methanol (10 mM), was cultivated in the medium of Heijthuijsen and Hansen (1989b). The strains were grown at 37°C except for the *Methanococcoides* strains (30°C), *Methanohalophilus zhilinaeae* (45°C) and *Methanobacterium* sp. Strain C8 (30°C).

**Analyses.** Headspace analyses of methane and methanethiol were performed by a slightly modified method of Visscher and van Gemerden (1991), in which a Supelpak S instead of a Porapak column was used. The concentration of methanethiol in the liquid phase was calculated using a distribution coefficient of 7.9 (Kiene and Capone 1988). In pure culture studies methane was measured by gas chromatography on a Porapak Q column with thermal conductivity detection (Heijthuijsen and Hansen 1989).

MMPA and mercaptopropionate were measured after esterification of 0.5 ml of sample (after centrifugation to remove cells or sediment) with methanol in sulfuric acid (50%) by the gas chromatographic method of Laanbroek et al. (1982) for analysis of lactate. Succinate was used as an internal standard. MMPA and mercaptopropionate were also analyzed by HPLC as described previously (van der Maarel et al. 1993).

**Chemicals.** MMPA was made by alkaline hydrolysis of its methyl ester (Aldrich, Steinheim, Germany) as described by Wackett et al. (1987). The identity and purity of the product was checked by $^1$H-NMR; the MMPA content was estimated by organic carbon analysis. Mercaptopropionate was obtained from Aldrich (Steinheim, Germany).

**Results**

**Enrichment culture.** After inoculation of mineral medium containing MMPA (20 mM) and yeast extract (50 mg/l) with anoxic marine sediment, MMPA was converted to methane, with methanethiol as an intermediate. After several transfers into fresh medium MMPA was still converted to methane, also when sulfate was omitted from the medium. The enrichment culture that was obtained in this way produced 14 mM methane from 20 mM MMPA. No methanethiol or acrylate could be detected. Epifluorescence microscopy showed large numbers of irregular coccoid cells which had a characteristic fluorescence at 420 nm. After treatment with the antibiotics ampicillin, vancomycin, and kanamycin (20 µg/ml final concentration each), which all act against bacteria but not against archaea, MMPA was still converted to methane and mercaptopropionate. When 2-bromoethanesulfonic acid (25 mM), a specific inhibitor of methanogenesis (Sparling and Daniels 1987), was added, no MMPA was converted to mercaptopropionate and methane. These observations made us speculate that methanogenic archaea present in the enrichment culture might have directly converted
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MMPA to mercaptopropionate and methane. Because of the morphological similarity of the methanogens present in the enrichment culture to coccolid *Methanosarcina* strains and the ability of *Methanosarcina* sp. strain MTP4 to metabolize methanethiol (Finster et al. 1992), a possible intermediate of MMPA degradation, we tested strain MTP4 for the ability to convert MMPA to mercaptopropionate and methane.

**Conversion of MMPA by pure cultures of methanogenic archaea.** *Methanosarcina* sp. strain MTP4 was found to be able to grow with MMPA as a substrate. A lag phase of approximately 7 days was observed when a methanol-grown culture was transferred to medium containing MMPA as a substrate. Transfer of a MMPA-grown culture to fresh medium with methanol or MMPA as a substrate gave no significant lag phase. During growth, MMPA was converted to mercaptopropionate and methane (Fig. 1A). The specific growth rate was 0.033 h⁻¹ (tₚ=21 h), based on the exponential production of methane between 51 and 119 hours. In a separate experiment, the conversion stoichiometry was determined; from 13.5 mM MMPA 13.5 mM mercaptopropionate and 10.2 µmol methane per ml medium were formed. The conversion corresponds to the following equation: 4 MMPA + 2 H₂O → 4 mercaptopropionate + CO₂ + 3 CH₄. The identity of the organic compound formed after growth of strain MTP4 on MMPA was established to be mercaptopropionate by [³H]-nuclear magnetic resonance and cochromatography (HPLC and gas chromatography) with authentic mercaptopropionate as a reference (data not shown).

Ten other methanogenic strains were tested for the ability to grow on MMPA. Only *Ms. acetivorans* DSM 2834 (Fig. 1B) and *Ms. siciliae* DSM 3028 (data not shown) were found to be able to grow on MMPA; the latter strain grew more slowly than strain MTP4 and *Ms. acetivorans*. *Ms. mazeii* DSM 3318, *Methanobacterium* sp. strain C8 DSM 3821, *Methanococcoides methylutens* DSM 2657, *Methanohalophilus zhilinaeae* DSM 4017, *Methanospirillum hungateii* DSM 864, *Ms. barkeri* strain Fusaro, *Ms. barkeri* strain MS, and *Methanococcoides* sp. strain PM2 did not grow with MMPA as a single substrate and did not convert MMPA during growth on their regular substrate (see Materials and Methods). The MMPA-utilizing methanogenic strains were unable to convert DMSP.

**Conversion of MMPA in sediment suspensions.** Methanethiol was formed within one day after addition of MMPA (500 µM) to a sediment suspension (Fig. 2A). The maximum concentration of methanethiol was approximately 200 µM; methanethiol started to decrease after two days. No methanethiol could be detected after 5 days. Methane formation started in the same period and reached its maximum after 5 days (417 µM). No methanethiol was formed in the presence of the antibiotics ampicillin, vancomycin, and kanamycin (Fig. 2B). Methane formation in these incubations was much slower than in the incubations without additions. The concentration of MMPA decreased at a rate similar to the increase in the concentration of methane. Methanethiol but not methane was formed when 2-bromoethanesulfonic acid (25 mM) was added to the suspension.
Fig. 1. (A) Growth of *Methanosarcina* sp. strain MTP4 on MMPA (13.5 mM). (B) Growth of *Ms. acetivorans* DSM 2834 on MMPA (10 mM). Symbols: ⬤, optical density at 430 nm (OD$_{430}$); V, methane; ●, MMPA; ■, mercaptopropionate. Cultures were grown in 120-ml crimp-seal bottles with 50 ml medium under an atmosphere of N$_2$-CO$_2$ (80/20 [vol/vol]). The methane line indicates the total amounts present in both the gas and liquid.
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Fig. 3 (A) Accumulation of methanethiol ($\square$) and methane ($\bullet$) in a sediment suspension after the addition of MMPA (500 µM). (B) Accumulation of methane ($\bullet$) in a sediment suspension after the addition of MMPA ($\square$) and antibiotics; no methanethiol was detected in the presence of antibiotics. Dotted lines are controls without MMPA addition. Sediment slurries (40 ml) were incubated in 70-ml crimp-seal bottles under a nitrogen atmosphere. Methane and methanethiol are the total amounts present in both the gas and liquid.

Discussion

This is the first report in which it is shown that a pure culture of a methanogenic archaeon can utilize MMPA as a substrate for growth. It adds to the limited number of compounds that are known as methanogenic substrates or electron donors for methanogenesis: H$_2$-CO$_2$, formate, CO, methanol, acetate, tri-, di-, and monomethylamine, dimethylsulfide, methanethiol, 1-propanol, 2-propanol, ethanol, 1-butanol, 2-butanol, 1,3-butadiol, cyclopentanol, and pyruvate (O’Brien et al. 1984; Oremland et al. 1989; Whitman et al. 1992; Finster et al. 1992; Bock et al. 1994; Rajagopal and LeGall 1994). The most important methanogenic substrates usually are H$_2$-CO$_2$ and acetate but in marine environments methylated compounds such as trimethylamine and dimethylsulfide are thought to predominate (Whitman et al. 1992). Methanosarcina strain MTP4 utilizes MMPA as a typical C$_1$-substrate and demethylates it to mercaptopropionate. Methanosarcina strain MTP4 was isolated from a salt marsh with methanethiol as the enrichment substrate (Finster et al. 1992); it can also grow on dimethylsulfide. Dimethylsulfide and methanethiol can be formed from methoxylated aromatics (Finster et al. 1990), but in the marine environment DMSP is most probably the major source of dimethylsulfide. DMSP is also a precursor of MMPA as suggested by sediment slurry experiments (Kiene and Taylor 1988a and b) and shown in pure culture studies with Desulfobacterium PM4 (van der Maarel et al. 1993). Thus, strain MTP4 originates from an environment where both dimethylsulfide and MMPA are present. Similarly, Ms. acetivorans DSM 2834 was isolated from marine sediment and is now known to metabolize both dimethylsulfide (Ni et al. 1994) and MMPA (this study). Ms. siciliae DSM 3028 was shown to be closely related to Ms. acetivorans (Ni et al. 1994).

The biochemical mechanism of MMPA demethylation by methanogens is still obscure. Wackett et al. (1987) showed that in crude cell extracts of H$_2$-CO$_2$-grown Methanobacterium thermoautotrophicum $\Delta$H MMPA, which is a structural analogue of methyl-S-coenzyme M, can serve as a substrate for the methyl-S-coenzyme M
reductase, an enzyme involved in the last step of methanogenesis (Ferry 1992). It is therefore possible that strain MTP4 is able to take up MMPA and use the methyl-S-coenzyme M reductase to convert MMPA. However, out of the eleven methanogenic strains tested only Methanosarcina sp. strain MTP4, Ms. acetivorans DSM 2834 and Ms. siciliae DSM 3028 were able to convert MMPA. It is therefore not very likely that MMPA utilization by strain MTP4, strain DSM 2834, and strain DSM 3028 is due to a general lack of specificity of the methyl-S-coenzyme M reductase. Alternatively, a MMPA:coenzyme M methyl transferase system might be used in the conversion of MMPA. Specific methyltransferases are known to be involved in the metabolism of methanol and methylamines, respectively (Ferry 1992).

The methanogenic conversion of MMPA to mercaptopropionate has also been found to occur in slurries prepared from anoxic marine sediments, but only when antibiotics were added. Under normal conditions, MMPA was readily converted to methanethiol and presumably acrylate. Methanethiol is further converted to methane by methanogenic archaea. These results suggest that in situ MMPA can serve as a substrate for methanogenic archaea but the major pathway for conversion might be demethiolation; it should be kept in mind, however, that at the low natural concentrations of MMPA the ratio between demethylation and demethiolation may be very different. Kiene and coworkers (Kiene and Taylor 1988a; Kiene et al. 1990) concluded that demethylation is a major transformation pathway for MMPA in intertidal sediments. They suggested that Eubacterium limosum-like bacteria might be responsible for the sequential demethylation of DMSP. Thus far, acetogenic bacteria that can demethylate DMSP have not been isolated. The combined activities of DMSP-demethyllating, sulfate-reducing bacteria (van der Maarel et al. 1993) and MMPA-demethylating methanogenic archaea may also be responsible for the observed conversion of DMSP to mercaptopropionate.

Cleavage of DMSP results in the formation of dimethylsulfide. Part of the dimethylsulfide escapes to the atmosphere, where it is oxidized to sulfuric acid and methanesulfonic acid (Charlson et al. 1987). These compounds may act as cloud condensation nuclei, and thus dimethylsulfide may exert a negative effect on global warming. Anaerobic metabolism of dimethylsulfide results in the formation of methane (Kiene et al. 1986; Finster et al. 1992), which can act as a greenhouse gas (Hogan et al. 1991), although part of the dimethylsulfide might be oxidized to CO$_2$ by sulfate-reducing bacteria (Kiene et al. 1986). Demethylation as well as demethiolation of MMPA can also result in the direct or indirect formation of methane. Part of the methane that is formed in the anoxic sediment can be oxidized in the upper oxic layer by methane-oxidizing bacteria but methane fluxes from salt marshes into the atmosphere have been found to exist (Senior et al. 1982). Therefore, we conclude that anaerobic demethylation of DMSP results in the production of a positive effector (methane) of global warming, whereas the cleavage into dimethylsulfide and acrylate leads to both a positive (methane) and a negative (dimethylsulfide) effector.

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