Carbon Isotope Fractionation during Acetoclastic Methanogenesis by Methanoseta concilii in Culture and a Lake Sediment

Holger Penning,1† Peter Claus,1 Peter Casper,2 and Ralf Conrad1*

Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch Str., 35043 Marburg, Germany,1 and Leibniz Institute of Freshwater Ecology and Inland Fisheries, Department of Limnology of Stratified Lakes, 16775 Stechlin, Germany2

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The isotope enrichment factors (ε) in Methanoseta concilii and in a lake sediment, where acetate was consumed only by Methanoseta spp., were clearly less negative than the ε usually observed for Methanosarcina spp. The fraction of methane produced from acetate in the sediment, as determined by using stable isotope signatures, was 10 to 15% lower when the appropriate ε of Methanoseta spp. was used.

Methane is a major product of the degradation of organic matter in anoxic environments like rice paddies, natural wetlands, and lake sediments. Its production from H2 and CO2 and acetate (ac) in natural systems has been quantified by stable isotope modeling (7, 17). One of the input parameters needed is the carbon isotope fractionation of acetoclastic methanogenesis. To determine the fractionation factor (α) in culture or in an environmental system, the δ13C of the methyl carbon of acetate (ac-methyl) must be known, since CH4 is produced from the methyl carbon rather than the carboxyl carbon of acetate (5, 19). For Methanosarcina spp. isotope fractionation has been determined in pure culture (7, 10, 20) and has been verified in natural environments (14). However, detailed data on isotope fractionation by Methanoseta spp. are rare. Valentine et al. (18) reported a rather low fractionation factor (α = 1.007) for thermophilic Methanoseta thermophila. Similarly, the fractionation factor for mesophilic Methanoseta concilii also seems to be lower (α = 1.017) (A. Chidthaisong, S. C. Tyler, et al., unpublished results) than that for Methanosarcina spp. (α = 1.021 to 1.027) (for a review see reference 18). A difference in isotope fractionation by the two acetoclastic methanogenic groups is possible, since they differ in their biochemical activation of acetate to acetyl coenzyme A (acetyl-CoA).

To increase our knowledge of isotope fractionation by Methanoseta spp., we grew M. concilii under defined conditions and determined the isotope enrichment factor (ε). We also determined isotope fractionation by acetoclastic Methanoseta spp. in a natural environment, Lake Dagow sediment, in which only members of the Methanosetaeae were detected (2, 5).

Cultures (n = 5) of M. concilii DSM 3671 were grown under N2-CO2 (80:20) at 37°C in glass bottles (250 ml; Müller-Krempe, Bülach, Switzerland) without shaking using carbonate-buffered (pH 7.1) mineral medium (DSM medium 334). Sodium acetate was added to an initial concentration of approximately 70 mM. For the total molecule the added acetate (ac) had a δ13C of −24.0‰, and for the methyl group the δ13C-ac-methyl was −29.4‰. After inoculation with a 20% bacterial suspension in the late exponential phase (resulting in a final volume of 125 ml), several gas (0.4-ml) and liquid (2-ml) samples were removed and used for analysis of pH, concentration, and the carbon isotope composition of acetate and the products formed.

Sediment samples from eutrophic Lake Dagow (Northern Brandenburg, Germany) were obtained on 9 November 2004 and 17 August 2005 and transported to the laboratory in Marburg as described previously (8). The sediment samples from 2004 (19 ml; depth, 0 to 10 cm) and from 2005 (10 ml; depth, 0 to 5 cm) were placed in 120-ml serum vials and 27-ml pressure tubes, respectively. The glass vessels were closed with butyl rubber stoppers and incubated under an N2 atmosphere at 10°C without shaking. After preincubation overnight, the appropriate volume of a sodium acetate stock solution (40.6 mM) was added to obtain an initial acetate concentration of 1 mM. The acetate had a δ13C of −32.1‰ and a δ13C-ac-methyl of −36.4‰. At each time examined during incubation triplicate tubes were analyzed and subsequently sacrificed. In another experiment, using sediment from 2005, CH4 production was investigated after consumption of the acetate that was added. To do this, the headspace was exchanged with N2, which was followed by repeated gas sampling over the next 18 days.

Chemical and isotopic analyses were performed as described by Penning and Conrad (13). A stable isotope analysis of 13C/12C in gas samples was performed using a gas chromatograph-combustion-isotope ratio mass spectrometer system (Thermoquest, Bremen, Germany) (13). Measurement of isotopes and quantification of acetate were performed with a high-performance liquid chromatography system coupled to Finnigan LC IsoLink (Thermo Electron Corporation, Bremen, Germany). Off-line pyrolysis was performed to determine δ13C-ac-methyl contents (1, 3).

The amount of total inorganic carbon (TIC) produced by M. concilii was calculated (13) and was expressed as the difference between the amount of TIC at any time and the initial amount of TIC. The isotope enrichment factor associated with aceto-
clastic methanogenesis was determined as described by Mariotti et al. (11) from the residual reactant

\[ \delta_i = \delta_{ai} + \varepsilon [\ln(1 - f)] \]  

(1)

and from the product formed

\[ \delta_p = \delta_{ai} - \varepsilon (1 - f)[\ln(1 - f)]/f \]  

(2)

where \( \delta_i \) is the isotope composition of the reactant (either ac or ac-methyl) at the beginning, \( \delta_r \) and \( \delta_p \) are the isotope compositions of the residual ac and the pooled CH\(_4\), respectively, at the instant when \( f \) was determined, and \( f \) is fractional yield of the products based on the consumption of ac (0 < \( f < 1 \)). Linear regression of \( \delta_r \) against \( \ln(1 - f) \) and of \( \delta_p \) against \( (1 - f)[\ln(1 - f)]/f \) gives \( \varepsilon \) as the slope. The enrichment factor was converted to the fractionation factor: \( \varepsilon = 10^\varepsilon \times (1 - \alpha) \).

The relative contribution of acetate- and CO\(_2\)-derived CH\(_4\) to total CH\(_4\) was determined as follows (4):

\[ f_{\text{ac}} = (\delta_{\text{CH}_4\text{-new}} - \delta_{\text{ma}})/(\delta_{\text{ac-methyl}} - \delta_{\text{ma}}) \]  

(3)

where \( f_{\text{ac}} \) is the fraction of CH\(_4\) formed from H\(_2\)-CO\(_2\), \( \delta_{\text{ma}} \) and \( \delta_{\text{ac-methyl}} \) are the isotope ratios of CH\(_4\) derived from either ac or H\(_2\)-CO\(_2\), and \( \delta_{\text{CH}_4\text{-new}} \) is the isotopic signature calculated for the methane formed since the last measurement (13). \( \delta_{\text{ma}} \) was calculated from \( \delta_{\text{ac-methyl}} \) using \( \alpha_{\text{ma}} \) (experimentally determined in this study and from previously published data). \( \delta_{\text{ac-methyl}} \) was calculated using the isotope fractionation factor for hydrogenotrophic methanogenesis (\( \alpha_{\text{CO}_2\text{-CH}_4} = 1.085 \)) determined previously for Lake Dagow sediment (2). For calculation of \( \delta_{\text{ma}} \), \( \delta_{\text{ac-methyl}} \) was assumed to be equal to \( \delta^{13}\text{C} \) of organic matter (\( \delta_{\text{org}} = -30.4 \pm 0.4 \)) (2).

During growth experiments with \( M. \text{concilii} \) acetate was consumed within 550 h to threshold concentrations and was converted to CO\(_2\) and CH\(_4\) (Fig. 1A). The amount of CO\(_2\) and CH\(_4\) accounted for approximately 73% of the initial amount of acetate. As expected for a closed-system approach, the \( \delta^{13}\text{C} \) values for both the substrate and the products increased due to continuous preferential consumption of the [\( ^{12}\text{C} \)] acetate (Fig. 1B). The initial high \( \delta^{13}\text{C} \) value of methane (\( \delta_{\text{CH}_4} \)) was due to transfer of dissolved CH\(_4\) by inoculation. \( \delta^{13}\text{C}_{\text{CO}_2} \) increased with time but was not used for determination of isotope fractionation, since the high background level of the bicarbonate buffer did not allow precise quantification of the \( \delta^{13}\text{C} \) of the newly formed TIC. Isotope fractionation during CH\(_4\) formation from acetate was determined from data for acetate, ac-methyl, and CH\(_4\) using equations 1 and 2 (Fig. 2). The isotope enrichment factors determined for ac-methyl (\( \varepsilon_{\text{ac-methyl}} = -10.4 \pm 1.5\%e \)) and CH\(_4\) (\( \varepsilon_{\text{CH}_4} = -9.4 \pm 0.9\%e \)) agreed within error, whereas the isotope enrichment factor for acetate (both carbon atoms) was more negative (\( \varepsilon_{\text{ac}} = -13.6 \pm 0.6\%e \)). The good correlation fit indicates that even for a wide range of concentrations (70 mM to the thresh-
old concentration) the magnitude of isotope fractionation was basically unchanged. Isotope enrichment in the carboxyl carbon of ac (ac-carboxyl) was calculated from \( \delta^{13}C_{ac-carboxyl} = 26^{13}C_{ac} - \delta^{13}C_{ac-methyl} \) using equation 1, resulting in an \( \epsilon_{ac-carboxyl} \) value of \(-16.0‰\).

Our study shows that the isotopic fractionation factor of acetate methyl to CH\(_4\) was much less in \( M. \) concilii (\( \epsilon = \sim -10‰\)) than in \( M. \) barkeri (\( \epsilon = \sim -20‰\)) (7). The agreement of isotope enrichment in ac-methyl (\( \epsilon_{ac-methyl} \)) (Fig. 2A) and CH\(_4\) (\( \epsilon_{CH4} \)) (Fig. 2B) is a reasonable result, since in \( M. \) concilii the methyl group should be almost exclusively (98%) used for methane production (12). After complete consumption of acetate, the intercept of the regression line of CH\(_4\) (\( \delta_{CH4} \)) (Fig. 2B) was \(-29.5‰\), which is almost identical to \( \delta^{13}C_{ac-methyl} \) (\(-29.4‰\)), as expected from equation 2. The \( \delta^{13}C \) of the biomass of \( M. \) concilii was not determined in the experiment reported here, but previous studies showed that the biomass was slightly \( ^{13}C \) enriched with respect to the initial \( \delta^{13}C_{ac} \) (\( \epsilon_{biomass} = 6.0‰ \pm 0.7‰ \)) (unpublished data). We assumed that due to the relatively low level of biomass formation in anaerobic metabolism \( \delta^{13}C_{CH4} \) was probably not significantly affected, but this might have resulted in slightly enhanced depletion of \( ^{13}C \) in the catabolic product CH\(_4\) (e.g., a lower \( \epsilon \) for CH\(_4\) than for ac and ac-methyl).

The depletion of \( ^{13}C \) in the ac-carboxyl (\( \epsilon_{ac-carboxyl} = -16.0‰\)) was greater than that in the ac-methyl, as observed previously for \( M. \) barkeri (7). The stronger fractionation in ac-carboxyl could theoretically have been caused by reversible exchange of the carboxyl carbon of acetyl-CoA with the \( ^{13}C \)-enriched CO\(_2\) in the growth medium. This exchange reaction was observed in \( M. \) barkeri (6). However, when we considered such an exchange reaction using a \( \delta^{13}C_{ac-carboxyl} \) value of \(-18.7‰\) of the initial acetate and a \( \delta^{13}C_{CO2} \) value of \(-24‰\) (Fig. 1B), we expected \( ^{13}C \) depletion leading to weaker isotope fractionation in \( \delta^{13}C_{ac-carboxyl} \), which was not the case. Therefore, the exchange reaction either might not be active in \( M. \) concilii or might be catalyzed to only a minor extent. As an alternative explanation we suggest that the stronger isotope fractionation in the ac-carboxyl was caused by \( ^{13}C \) fractionation during conversion of acetate via acetyl phosphate to acetyl-CoA. The actual bond breaking of this reaction occurs at the carboxyl carbon (5), and therefore the carboxyl carbon might experience a stronger isotope effect.

The isotope fractionation during acetoclastic methanogenesis in \( M. \) thermophila in the study of Valentine et al. (18) is apparently weaker than that in \( M. \) barkeri spp., in which fractionation factors typically range from 1.021 to 1.027, equivalent to enrichment factors of \(-21\) to \(-27‰\) (7, 10, 20). Gelwicks et al. (7) proposed that the carbon monoxide dehydrogenase (CODH) enzyme complex catalyzes the rate-limiting step of the overall reaction and therefore is responsible for the observed fractionation. Yet this does not explain the difference in isotope fractionation caused by the two groups of acetoclastic methanogenic archaea. Although the CODHs of \( M. \) thermophila spp. and \( M. \) barkeri spp. differ to some extent, the enzymes catalyze the same principal reactions, which determine the magnitude of the isotope effect. Thus, a priori it is not expected that the two CODHs would exhibit very different isotope effects. We therefore speculated that the difference in the overall isotope fractionation is caused by the initial activation of acetate. In this reaction the two acetoclastic genera differ biochemically. While \( M. \) thermophila spp. activate acetate in two steps (acetate kinase and phototransacetylase) at the expense of one high-energy phosphate bond, \( M. \) barkeri spp. activate acetate using the acetyl-CoA synthetase at the expense of two high-energy phosphate bonds (9). Activation by \( M. \) thermophila spp., which is driven only by the energy from the breakage of one high-energy bond, may have greater reversibility. This could explain why the isotope effect of the later C—C bond cleavage of acetyl-CoA is expressed more strongly.
Lake Dagow sediment (obtained in 2004) was incubated anaerobically after addition of acetate (initial concentration, 1 mM). Under these conditions CH4 was produced from H2-CO2, from acetate that is naturally produced in the sediment, and from the acetate added (Fig. 3A). Within 92 h, the elevated concentrations caused by addition of acetate decreased to constant low concentrations (93 ± 18 µM). Quantification of acetate and CH4 showed that in the sediment samples 52% of the total CH4 produced was derived from the additional acetate. Note that acetoclastic methanogenesis was the only acetate-consuming process in the sediment, which was devoid of sulfate or other inorganic electron acceptors (2). Due to preferential consumption of 13C for CH4 production, ac, as well as ac-methyl, became enriched in 13C with time (Fig. 3B). Simultaneously, CH4 also became isotopically enriched, but to a minor extent. After 50 h, 613C again decreased due to dilution of the residual 13C-enriched acetate by acetate produced from anaerobic degradation of sediment organic matter. Since CH4 was also produced from H2-CO2, isotope enrichment by acetoclastic methanogenesis was calculated only from the isotope data for acetate. Only data for f values that were ≥0.15 and ≤0.5 (ln(1 − f) = −0.7) were analyzed, since acetate was still being produced in the sediment. At a higher f value the continuously produced isotopically relatively light acetate would dilute δ13Cac-methyl and δ13Cac (Fig. 3) and therefore bias the quantification of ε. The values of εac and εac-methyl determined were −12.9‰ ± 1.2‰ and −14.2‰ ± 2.2‰, respectively. The data point at a ln(1 − f) value of 0.69 influenced the determination of ε. Removal of this data point altered εac to −20.3‰, yet εac-methyl changed only slightly to −11.6‰. We also studied sediment obtained in 2005. Although the ε values determined were somewhat different (εac = −17.2‰ ± 1.2‰ with r2 = 0.928; εac-methyl = −7.8‰ ± 1.8‰ with r2 = 0.566), they clearly confirmed the tendency of ε to be greater than −20‰. Notably, the results for Lake Dagow sediment confirmed that the ε values for Methanoseta spp. were clearly less than those for Methanosaeta spp. (7, 10, 20).

The difference between the two acetoclastic methanogenic genera is important for correct computation of the amount of CH4 that is produced from acetate compared with the amount of CH4 that is produced from CO2 (equation 3). A lower εac-methyl value leads to a higher value of fmc. Thus, application of the fractionation factor of Methanosaeta spp. to an environment that is dominated by Methanosaeta spp. would result in underestimation of the relative contribution of H2-CO2-dependent CH4 production. We tested the influence of εac-methyl on this quantification using data from anoxic incubation of Lake Dagow sediment. We used sediment samples from 2005 and calculated the relative fraction of CH4 produced from H2-CO2 (fmc) (equation 3) during the incubation while CH4 was produced at a constant rate (Fig. 4A). 613C_CCO2 in the carbonate-buffered system stayed fairly constant, while δ13C_CH4 decreased, reaching stable values around −63‰. The sensitivity of fmc to εac-methyl was calculated using the enrichment factors either for Methanosarcina spp. (7), for M. concilii (Fig. 2A), or for Lake Dagow sediment (Fig. 4B). Comparison of calculated fmc values showed that fmc was on average larger by 0.11 units (εac-methyl = −14.2‰) and 0.15 units (εac-methyl = −10.4‰) using enrichment factors obtained in this study rather than using previously published values typical for acetoclastic Methanosaeta spp. (εac-methyl = −20‰). The final fmc values were 0.33, 0.42, and 0.46 for εac-methyl values of −20‰, −14.2‰, and −10.4‰, respectively. The fmc values of 0.2 to 0.3 calculated for an previous incubation experiment performed with Lake Dagow sediment (2) might have been biased by such an inappropriate fractionation factor, so that the real values probably are slightly higher (0.3 to 0.4), as suggested by Fig. 4.

Our results show that two functionally equivalent groups of acetoclastic methanogens, whose biochemical pathways differ, exhibit different isotope fractionation and consequently affect isotope modeling. Hence, microbial community structure can affect biogeochemical fluxes and thus provides an example of the hypothesis that the composition of environmental microbial communities does affect ecosystem function (15, 16).

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