Understanding the stable isotope composition of biogenic methane in laboratory settings and in the environment

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Methane (CH_4) is a major greenhouse gas, with natural and anthropogenic sources (*1*). The main natural source of methane is microbial methanogenesis—the metabolic activity of Archaea inhabiting anoxic environments (*2*). Strong discrimination against the heavy isotopes of hydrogen and carbon associated with methanogenesis has led to the wide use of the isotope ratios of hydrogen (D/H) and carbon ($^{13}C/^{12}C$) in methane, and recently also methane clumped isotopes (*3*–*6*), to trace its source (*7*) and to investigate the processes by which it is formed and consumed. The insight gained is crucial for construction of the global methane budget, and for assessment of the climatic and environmental effects of methane in Earth's past, present and future. Isotopic information may only be used to constrain the methane budget if the processes that fractionate hydrogen and carbon are well understood, however, as these processes often have overlapping signatures, this interpretation is limited. The wide range of biogenic methane isotopic signatures was hypothesized to be controlled by the Gibbs free energy of methanogenesis (ΔG[1]), due to differential reversibility of the enzymatically-catalyzed reactions in the pathway (*8*), but this was not proven yet. Previous attempts to model isotope fractionation in methanogenesis with simplified metabolic models demonstrated that some of the observed fractionation range can be explained by bio-isotopic models (*5*, *9*, *10*), but they failed to describe the entire range, and more importantly, the relation to ΔG. Bio-isotopic models link cellular metabolism with isotopic fractionation, by predicting the pathway reversibility degree utilizing large sets of biochemical and isotopic parameters. These models reveal complex environmental and metabolic controls over isotopic fractionation (*5*, *11*,

[1] In this report we use ΔG instead of $-\Delta G_r$, for brevity.

12), that complement experimental and environmental studies. Here we describe the development and results of a bio-isotopic model of methanogenesis, that can predict the isotope fractionation of C, H and clumped isotopes and their relation to ΔG. We also extend our model to natural environments, where methanogenesis and biological methane consumption (methanotrophy) cooccur. In these environments, methane often shows a trend of isotope 'aging', for H and clumped isotopes (*13*, *14*). We describe a simplified three-box model of methane cycling, that captures C, H and clumped isotope evolution in natural anoxic environments.

1. Model development

We developed a bio-isotopic model for the hydrogenotrophic methanogenesis pathway, where dihydrogen (H_2) derived electrons reduce carbon dioxide (CO_2) to methane. The model ultimately predicts C and H isotope ratios and clumped methane isotopologues ($\Delta^{13}CH_3D$) and their relation to ΔG of methanogenesis. The model is based on the general framework developed for sulfate reducing bacteria, linking the thermodynamic drive with the isotopic fluxes (*11*, *15*). It is applicable to many biological systems, assuming all enzymatically catalyzed reactions in the pathway are potentially fully reversible, and that the metabolites are soluble within a single cellular compartment. We assign a reversible Michaelis-Menten type rate law with thermodynamic and kinetic components (*16*) to each of the reactions in the pathway, including electron carrier cycling and CO_2 diffusion through the membrane (see Fig. 1, left, and supplementary materials), and find the metabolites' steady state concentrations by solving a set of ordinary differential equations. We compiled the kinetic and thermodynamic parameters from the literature, and randomly sampled unknown parameters from distributions that represent their uncertainty and fitted the value of the net metabolic flux to minimize the sum of squared errors (SSE) for any given set of parameters.

The metabolites concentrations set the reversibility degree of each enzymatically catalyzed reaction (f), through the relation $f = e^{\Delta G/RT}$ (*17*), which dictates the extent of kinetic vs. equilibrium isotope effects for individual reactions. This drive can also be related to the ratio of the backward

and forward metabolic fluxes (J) by $f = J^{-}/J^{+}$. Biological pathways can approach equilibrium if the backward rate is high enough, and, in this case, f approaches unity. If the forward rate far exceeds the backward rate, the reaction is practically unidirectional, and f approaches zero.

To find the isotope compositions of the metabolites, we applied an isotopic mass balance, considering the reversibility and isotopic flux of each reaction and assigned kinetic and equilibrium fractionation factors (KFFs and EFFs, respectively). As most of these parameters were not determined experimentally, we used EFFs calculated by quantum mechanical approaches (*18*), and sampled KFFs from random distributions, and fitted them to isotopic fractionation observation, to correlate the isotopic compositions with ΔG.

2. Metabolic model results – $\Delta G_r'^0$ controls initial departure from equilibrium

To validate our metabolic model, we first demonstrated that it can predict the observed relation of cell-specific methanogenesis rate and H_2 concentrations (Fig S.3) (*19*). We then used the metabolites concentrations (Fig S.2) to determine the degree of reversibility, and found that the main control over the departure from equilibrium is the standard Gibbs free energy ($\Delta G_r'^0$) of the reactions (Fig. 1, right), as the first reactions to depart from equilibrium have the largest-negative $\Delta G_r'^0$ (see Table S.2), and the reactions' half-saturation constants (K_M) has a secondary control (see supplementary section S.X). Overall, the entire pathway becomes more favorable with the increase in ΔG, yet each reaction has a distinct trajectory for the departure from equilibrium. Some reactions, like Frh-catalyzed F_{420} reduction, do not fully depart from reversibility in the ΔG space we explored. The Mvh/Hdr, Mcr and Mtr-catalyzed reactions depart from reversibility at ΔG<15 kJ mol^{-1}, followed byΔG Ftr- and Fmd-catalyzed reactions (ΔG ≈40 and 70 kJ mol-1, respectively).

The metabolic model provides insight into the dynamics of the hydrogenotrophic pathway's departure from equilibrium with increasing ΔG. For example, the first step in the pathway is CO_2 fixation to organic carbon, catalyzed by Fmd ($\Delta G_r'^0 = +10.3$ kJ mol^{-1}). To drive the pathway in the

direction of net methanogenesis, ferredoxin (Fd) needs to be ~100% reduced (i.e., reduced to oxidized ferredoxin ratio, $R_{r/o}^{Fd}$, of ~100). $R_{r/o}^{Fd}$ depends mainly on [CO_2], and it generally increases with a decrease in [CO_2], to compensate for the loss of thermodynamic drive. The redox state of cofactors F_{420} (F_{420}) and coenzyme B (HS-CoB) is smaller at low ΔG, with $R_{r/o}^{F_{420}}$ and $R_{r/o}^{CoB}$ of 10^{-3} and 10^{-1} respectively, and they both depend on H_2 concentrations, similar to previous observations (*20*). The concentrations of the metabolites of the main pathway generally mainly depend on [H_2], yet they show inverse trends: while the first three metabolites, CHO-MFR, CHO-H_4MPT and CH-H_4MPT decrease with [H_2] increase, the last three, CH_2-H_4MPT, CH_3-H_4MPT and CH_3-S-CoM, increase together with [H_2]. [CO_2] has only a marginal effect on these metabolites.

3. Mcr-Mtr and Ftr-Fmd KFFs control carbon isotope fractionation

Methanogens grown in laboratory cultures show an inverse correlation of CO_2-CH_4 fractionation ($ln^{13}\alpha_{CO_2\text{-}CH_4}$) and ΔG of methanogenesis, in the range ~20-90‰ (*8*, *14*, *21–23*) (Fig 2a). We found that when $\Delta G \to 0$, the individual reactions in the pathway proceed almost at equilibrium and predicted $ln^{13}\alpha_{CO_2\text{-}CH_4}$ is the equilibrium fractionation alone (Fig 2b). When ΔG slightly increases, C fractionation peaks to larger-then-equilibrium values of 80-100‰ at ~45 kJ mol^{-1}, due to a combination of kinetic isotope effects (KIEs) by Mcr and Mtr and the equilibrium isotope effects (EIEs) of the upstream reactions. This arises from the inherent properties of isotope fractionation systematics in linear metabolic pathways, where the net fractionation from reactant to product is dependent on the departure of reactions from chemical equilibrium (See section S.1.1.1). A unidirectional reaction will cause an isotopic reservoir effect, consuming all the reactants, thus inhibiting expression of downstream reactions fractionation. With ΔG further increasing, the Ftr- and Fmd-catalyzed reactions also depart from reversibility, and their KIEs defines the lower C fractionation boundary at ~115-160 kJ mol^{-1}. In maximal methanogenesis flux, CO_2 diffusion through the membrane becomes limiting, and methanogens consume intracellular CO_2

quantitatively. In this case, the minimal $ln^{13}\alpha_{CO_2\text{-}CH_4}$ of ~10‰ is a manifestation of CO_2 membrane diffusion reversibility, and the downstream fractionation inherited from Fmd and Ftr. As [CO2] decrease, so does $ln^{13}\alpha_{CO_2\text{-}CH_4}$ (Fig. 2c). This may explain the observations of the minimal $ln^{13}\alpha_{CO_2\text{-}CH_4}$ in hyperthermophilic--high pressure environments (*22*).

4. Large KFFs dominate hydrogen fractionation

Fractionation from CH_4 to H_2O in methanogenesis is due to preferential uptake of light protons from intracellular H_2O during C atoms reduction, through the electron donors Fd, F_{420} and HS-CoB. In laboratory cultures, CH_4-H_2O fractionation ($ln\,^2\alpha_{CH_4\text{-}H_2O}$) is usually in the range ~–200 to –600‰, and there is a weak negative relation with ΔG (*8*, *9*, *14*, *23–25*) (Fig. 2d). We modeled the steady state isotope fractionation using a mass balance that accounts for the stoichiometry of the addition reactions in a branched metabolic network. We found that as Mvh/Hdr and Mcr depart from equilibrium, in the ΔG range of 0 to 30 kJ mol^{-1}, $ln\,^2\alpha_{CH_4\text{-}H_2O}$ decreases from equilibrium values to ~–450‰ (Fig 2e). Further departure from equilibrium of the pathway has a smaller overall effect on $ln\,^2\alpha_{CH_4\text{-}H_2O}$, evident from the small changes in the range 40 to 170 kJ mol^{-1}. Overall, in most experimental conditions, $ln\,^2\alpha_{CH_4\text{-}H_2O}$ is ranging between –350 to –450‰, and the effects of differential departure from equilibrium are limited.

The enzyme Hmd can directly deliver H atoms from H_2 to methylene-H_4MPT, in parallel to the Mtd-catalyzed reaction (Fig 1, left), introducing a potential isotopic fractionation between CH_4 and H_2 (*14*, *25*), if the intracellular H_2 pool is at isotopic disequilibria with H_2O. Specifically, spiking a methanogenic culture with D_2O or HD produced $ln\,^2\alpha_{CH_4\text{-}H_2O}$ in the range of –160 to -650‰ at ΔG=~135‰ kJ mol-1. The degree of $ln\,^2\alpha_{CH_4\text{-}H_2O}$ was mostly dependent on the initial fractionation between H_2O and H_2 (*14*). We found that the Hmd activity in high ΔG promotes the expression of the isotope composition of H_2 (δD_{H_2}), due to the direct incorporation of H_2-derived hydride ions.

If the initial H2O-H2 fractionation (${}^{2}\alpha_{H_2O(l)-H_2}$) is lower than the equilibrium H_2O-H_2 isotope fractionation, (${}^{2}\alpha^{eq}_{H_2O(l)-H_2}$=3.14 at 60°C (*26*)), $ln\ {}^{2}\alpha_{CH_4\text{-}H_2O}$ is expected to be less negative, whereas for ${}^{2}\alpha_{H_2O(l)-H_2}$ larger than ${}^{2}\alpha^{eq}_{H_2O(l)-H_2}$, $ln\ {}^{2}\alpha_{CH_4\text{-}H_2O}$ will be more negative (Fig. 2f). As Hmd and Mtd activities are mutually exclusive, depending on H_2 availability, this effect is more pronounced at high ΔG, or in exponential growth phases (*27*, *28*).

5. KIFs exert early onset of departure from equilibrium clumped signature

Clumped CH_4 isotopologues ($^{12}CH_2D_2$ and $^{13}CH_3D$) provide a sensitive tool to measure methane formation temperatures. While initially purposed as a geo-thermometer, it is now clear that source mixing (*29*) and vital effects (*5*) often disrupt this relation, especially in biogenic dominated environments. Moreover, so far, there have been no reports of microbial laboratory culture that produces an the temperature dependent equilibrium $\Delta^{13}CH_3D$ signal (*30*). Our model predicts that $\Delta^{13}CH_3D$ rapidly decreases from its equilibrium value with ΔG increase (Fig. 2g), with Mvh/Hdr, Mcr- and Mtr-catalyzed reactions departure from equilibrium, introducing ~4.5‰ anti-clumping effect, afterwards $\Delta^{13}CH_3D$ plateaus between ΔG=40 to 100 kJ mol^{-1}. With the other reactions departing from equilibrium at ΔG>100 kJ mol^{-1}, $\Delta^{13}CH_3D$ further decreases to its minimal value. The minimal and negative $\Delta^{13}CH_3D$ value in high ΔG is a combination of Mcr, Mtd, Mer and Fmd ${}^{13,2}\gamma_{kin}$. As we have no constrains for $\Delta^{13}CH_3D$ relation to ΔG in lab setups, we resorted to its relation to $ln\ {}^{2}\alpha_{CH_4\text{-}H_2O}$ (*5*, *31*). We note a considerable variation between hydrogenotrophic methanogens with and without cytochromes, the latter are the focus of our model. While $ln\ {}^{2}\alpha_{CH_4\text{-}H_2O}$ is of similar range, $\Delta^{13}CH_3D$ for methanogens with cytochromes is in the range of –6 to 0‰, while for methanogens with cytochrome the observed range is –2 to 3‰. The data collected so far in lab experiments is not sufficient to determine whether this is statistically significant, yet there may be physiological explanations for that, as methanogens with cytochromes have some distinct metabolic characteristics that may affect the dynamics departure from equilibrium. In lab cultures,

the gradual departure from equilibrium is expressed in both H and clumped isotope systems as a gradual shift to more negative values. The observations are limited to $ln\ ^2\alpha_{CH_4\text{-}H_2O}$<400‰ and $\Delta^{13}CH_3D$<2.5‰, which represent growth in high ΔG. Future experiments in low ΔG should yield the entire range, yet it is reasonable to assume that as for $ln\ ^2\alpha_{CH_4\text{-}H_2O}$, reaching full equilibrium $\Delta^{13}CH_3D$ values will be challenging.

6. Modeling methane cycling in natural environments

So far, we described a mechanism for isotope fractionation in lab cultures, yet there is much interest in applying this approach to natural environment. Methanogenesis in natural environments usually concurrent to methane oxidation, mitigating the full potential of methane emissions to the atmosphere. In anaerobic sediments, sulfate driven anaerobic methane oxidation (AOM) consumes ~45-61 Tg methane annually, making sulfate driven AOM a quantitative sink for methane produced below the sulfate-methane transition zone (SMT) (*32*). Consequently, the isotopic signal of methane is a combination of both methane production and consumption, with a typical trend of an approach towards isotope equilibrium with aging (Fig. S.3). This tendency towards equilibrium was explained either by (*i*) very slow methanogenesis rates, due to severe H_2-limitation (*5*, *33*), or by (*ii*) methane cycling during AOM, as the activation of methane in AOM by Mcr is probably acting close to equilibrium, suggesting a back flux between H_2O and methane (*13*, *14*, *34*). We developed a mass-balance model that predicts isotope composition evolution in anaerobic environments, by prescribing the ratio of rates of AOM and methanogenesis, $R_{MT/MG}$, and cycling between the intracellular methane and the methyl precursor pools. We prescribed the starting conditions to represent typical isotope compositions of young methanogenic pools, and follow the deviation from the expected temperature-dependent equilibrium fractionation, to normalize the effect of temperature effect. For bulk C and H isotopes, our model distinguishes between three regimes of isotopic evolution during methane aging in natural environments, controlled by $R_{MT/MG}$ (Fig. 3): (*i*) when $R_{MT/MG} > 1$, both C and H isotopic fractionation decrease with time (Fig. 3c).

For methane distillation, i.e., no cycling, fractionation decreases linearly, and depends on the KFF during methane activation. (*ii*) When $R_{MT/MG} < 1$, methane cycling promotes H equilibration, but increases C fractionation (Fig. 3a). With less cycling, H fractionation decreases because of kinetic isotope effect between CH_3 to CH_4. (*iii*) When $R_{MT/MG} = 1$, both C and H converge with equilibrium values, and cycling drives faster H isotope equilibration (Fig. 3b). Clumped isotopologues represent the internal ordering of C-H bonds in methane. Biogenic samples from laboratory cultures and young environmental samples diverge from internal equilibria by up to 8‰, always in the direction of 'anti-clumping'. Our cycling model shows that as in bulk C and H isotope systems, rapid cycling between the methane and methyl precursor pools promotes equilibration, and similar methanogenesis and methanotrophy rates will do the same (i.e., $R_{MT/MG} = 1$). In regimes of $R_{MT/MG} < 1$, equilibration is only obtained with rapid cycling of ($nR > \sim 10$).

Biogenic methane isotope composition in 'aged' reservoirs, such as coalbed methane and marine sediments, can thus be explained as maturation of lighter, 'younger', methane due to methanotrophy and methane cycling, in regimes where methanogenesis outcompetes or is similar to methanotrophy (i.e., $R_{MT/MG} \geq 1$). Our cycling model does not rule out extremely slow methanogenesis, yet we suggest that this explanation is unlikely. Our bio-isotopic model shows that electron carrier cycling by Mvh/Hdr is essentially irreversible throughout the entire ΔG range, inhibiting complete equilibrium of H_2O and CH_4 by generating D-depleted HS-CoB. CH_3-CoM reduction by Mcr is also rapidly departing from equilibrium, 'blocking' C isotope fractionation. While, in theory, a fully reversible pathway is possible, we hypothesize that basal power requirement, due to processes such as protein degradation and ion leakage (*35*), drive the methanogenic pathway out of equilibrium, even in energy-limited environments. Methanogenesis in extremely energy-limited environments should result in equilibrium signals of C and H isotopes and clumped isotopologues altogether. However, apparent equilibrium between CO_2 and CH_4 is

rare in the data we compiled, supporting the hypothesis of methane cycling as a primary control over isotope composition in anaerobic environments, and not extremely slow respiration rates.

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