Estimation of methanogenesis by quantification of coenzyme F430 in marine sediments

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(Received April 10, 2015; Accepted December 2, 2015)

Coenzyme F430 is the hydrocorphinoid nickel complex which acts as active site in methyl-coenzyme M reductase (MCR). The MCR-F430 complex catalyzes the last step of methanogenesis: reduction of methyl-coenzyme M to methane. Since F430 is a common enzyme for methanogens, it can be a function-specific biomarker to estimate biomass and activities of methanogens in environmental samples. A recently developed high sensitive method for coenzyme F430 analysis and a further purification step revealed F430 concentrations at 69 and 88 meters below seafloor (mbsf) off Shimokita Peninsula (529 and 31.3 fmol g-wet–1) and at 60 and 275 mbsf at Nankai Trough (31.4 and 26 fmol g-wet–1). Estimated methanogenic biomass and activities based on reported F430 content and activities in methanogens and prokaryotic cell weight, were ranging from 3.9 ¥ 10⁶ to 2.7 ¥ 10⁷ cells g-wet–1 and from 1.7 to 11 mmol d–1 g-wet–1.

Keywords: coenzyme F430, methane hydrate, methanogens, function-specific compound analysis, marine sediment

INTRODUCTION

During the last four decades, methanogenesis in marine sediments has been intensively studied because of its importance to understand carbon cycle on the Earth surface. So far, the studies revealed that methane is mainly of biogenic rather than abiogenic origin among surface to several hundred meters below seafloor (Kvenvolden, 2002; Milkov, 2004). The biogenic methane is produced by methanogenic archaea (methanogens) which utilize C₁ and C₂ compounds such as carbon dioxide, acetate, or methanol for carbon substrates.

The carbon substrates for methanogenesis are produced during the degradation of sedimentary organic matter by a sequence of microbial processes (Whiticar., 1999). Since the methanogenesis is the last step of the degradation of organic matter in anaerobic environments, investigation of their quantitative distribution and activities of methanogens are important to understand global carbon cycles (Thauer et al., 2008).

The microbially derived methane can be a major constituent of methane hydrate at continental margin. The globally deposited methane hydrate is estimated to be ~100,000 Gt (Kvenvolden, 2002; Milkov, 2004), which is a potent energy resource. Since the major source of methane in the hydrate is of methanogen origin, investigation of distribution and activities of methanogens leads to deeper understanding of the starting point of methane hydrate formation as well as migration and accumulation of methane.

In subsurface marine sediments, available techniques to detect methanogen’s activity are limited. Molecular biologic techniques have been used to investigate microbial community structure and function-specific gene analysis. However, in many cases methanogen have not been detected in marine sediments although methanogenesis is a major metabolic process in anoxic subseafloor sediments, suggesting either a low population of methanogens or unknown methanogens which cannot be detected with available primers (Biddle et al., 2006; Fry et al., 2008; Valentine, 2011). Analyses of archaeal membrane lipids and their isotopic composition are powerful tools to estimate biomass and source organisms. However, any membrane lipid species are not representative for methanogens. Known constituents of membrane lipids of methanogens are archaeol, cardoarchaeol, sn-2- and sn-3-hydroxyarchaeols, and macrocyclic archaeol (Koga et al.,...
Recent we developed analysis of coenzyme F430 by coenzyme F430 is the hydrocorphinoid nickel complex which acts as active site in methyl-coenzyme M reductase (MMR). This coenzyme complex catalyzes the last step of methanogenesis: reduction of methyl-coenzyme M to methane. Since F430 is a common functional specific compound in methanogenic pathways including hydrogenotrophic, acetotrophic and methylotrophic methanogenesis (Thauer, 1998), all methanogens including uncultured methanogens should utilize F430 for methanogenesis. Recent studies suggested that anaerobic methane oxidizing archaea (ANME) also utilize F430 and its homologue for the reversed methanogenesis (Krüger et al., 2003; Mayr et al., 2008; Shima et al., 2012). Thus, F430 is a function-specific compound for both methanogenesis and anaerobic methane oxidation, which has a potential to be a practical biomarker compound for estimation of biomass and activity of methanogens and ANME in subsurface environments.

Recently, we have developed a highly sensitive method for the analysis of F430 in the environmental samples with an on-line liquid chromatography-mass spectrometry. The triple quadrupole mass spectrometer enables F430 detection as low as 0.1 fmol. It corresponds to 6 attomoles of F430. The LC-MS/MS system (Kaneko et al., 2014) was supplied from Sumitomo Heavy Industry Ltd., Yokosuka, Japan.

Two sediment samples were collected from 69 and 88 m below the seafloor (mbsf, Site 9001; core section 8-4 and 10-4) off the Shimokita Peninsula, Japan (41°10.6380′ N, 142°12.08′ E) during the shakedown cruise CK06-06 of the D/V Chikyu (Aioke, 2007).

Another two sediment samples were collected from 60 and 275 mbsf (Site 0004, Site 29R2) at Nankai Trough (33°13.2278′ N, 136°43.3312′ E) during expedition 316 of Integrated Ocean Drilling Program (IODP, Kinoshita et al., 2009).

**Materials and Methods**

**Sample description**

A granule sample consisting of high-density methanogenic archaea including genus Methanobacterium, Methanoseta and Methanomicrobium (code MBK, Kaneko et al., 2014; Takano et al., 2013) was supplied from Sumitomo Heavy Industry Ltd., Yokosuka, Japan.

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**Extraction of F430**

Samples (wet) were extracted with 1% formic acid (pH 2; Wako Pure Chemical Industries Ltd.) by ultrasonication for 30 min on ice, followed by centrifugation (×10,000 g; 30 min at 4°C) to recover the supernatant. This step was repeated three times. The combined supernatant was introduced to an anion exchange column (Q Sepharose column; GE Healthcare) that had been equilibrated with 50 mM of Tris/HCl (pH 7.5) and washed with deionized water prior to use. The recovered eluent was introduced to a C18 SPE column (Sep-Pack; Waters Corp.) that had been equilibrated with methanol (overnight) and conditioned with 5 mL of 1% formic acid. An absorbed yellowish band (F430 fraction) on the column was eluted with 100% methanol. The recovered F430 fraction was dried and stored at –20°C prior to further treatment.

**Quantification by liquid chromatography-mass spectrometry (LC-MS/MS)**

LC-MS/MS was performed using an Agilent HPLC 1260 Infinity coupled to a 6460 Triple Quadrupole (QQQ) LC/MS system (Kaneko et al., 2014). Briefly, F430M was analyzed in positive ion mode by electrospray ionization (ESI) with an Agilent JetStream. Source and sheath gas flow rates were set to 5 and 11 L min⁻¹, respectively. Capillary and nozzle voltages were
set at 3500 and 500 V, respectively. For multiple reaction monitoring (MRM) analysis, the fragmentor voltage was 180 V and the collision energy was 0 V (zero-collision energy MRM, Kaneko et al., 2014), which is substantially SIM (selective ion monitoring)–SIM analysis. Both precursor and product ions of F430 were set to m/z 975.4 for F430M, 1021.4 for methylthio-F430. Compound separation by HPLC was conducted using a ZORBAX Eclipse XDB-C8 (4.6 x 250 mm; 5 µm p.s., Agilent Technologies). Mobile phases were 10 mM ammonium acetate (A) and acetonitrile (B). The flow rate was 0.5 mL min⁻¹. The gradient condition was started at 0% B followed by 30% B after 3 min and then 90% B after 90 min.

Concentration of coenzyme F430 was calculated by external standard method using concentration known coenzyme F430 standard. Coenzyme F430 for the standard was extracted from methanogenic granule and purified with a preparative HPLC. Purity and concentration of the standard solution was determined by nuclear magnetic resonance spectroscopy (NMR) and isotope ratio mass spectrometry coupled with elemental analysis (EA-IRMS) in earlier study (Kaneko et al., 2014). The concentration of the coenzyme F430 standard is 0.79 ± 0.03 µmol mL⁻¹.

Silica gel column chromatography
Silica gel column chromatography was performed for the F430M fraction to remove organic matrices in the sample. Silica gel (Sigma-Aldrich, 200–400 mesh) was activated by combustion at 450°C for 5 h, then deactivated with 1 wt% H₂O prior to use. Silica gel (1.4 mL) stored in hexane solution was loaded to a grass tube (6 mm i.d.) and conditioned with 3 bed volume of methanol. The F430M fraction dissolved in methanol was gently loaded on the silica gel column. The column was washed with 3 mL (2 bed volume) of methanol (F1) and acetoniitrile (F2), followed by elution of F430M (F3) with 3 mL (2 bed volume) of 100 mM NaClO₄ in acetoniitrile/ H₂O (80/20 v/v). F3 fraction was dried under N₂ stream. Recovery of F430M and removal of NaClO₄ was simultaneously performed by liquid-liquid extraction with H₂O and dichloromethane.

RESULTS AND DISCUSSION
Application in marine sediments
Presence of methanogens in sediments off Shimokita Peninsula has been suggested in previous studies. Methanogens belonging to the genus Methanobacterium, Methanosarcina, Methanococcoides, and Methanobrevibacter have been isolated from sediment at a depth of 107 m using a down-flow hanging sponge (DHS) reactor (Imachi et al., 2011). In our earlier study, F430 was also detected at 97 and 107 mbsf (Kaneko et al., 2014; Takano et al., 2013).

In this study, we investigated distribution of F430 at four sedimentary layers (69 and 88 mbsf off Shimokita Peninsula and 60 and 275 mbsf at Nankai Trough). In all sediments, F430 was successfully detected (Fig. 1). In particular, F430 concentration at 69 mbsf is 530 fmol g⁻¹ wet sediment, which is 10 times higher than those reported in our previous study (60 fmol g⁻¹ wet⁻¹ at 97 mbsf and 40 fmol g⁻¹ wet⁻¹ at 107 mbsf, Table 1).

Since the concentration of sedimentary F430 can reflect methanogens biomass in situ based on observation in nature (Kaneko et al., 2014), high concentration of F430 at 69 mbsf suggests a more abundant population of methanogens relative to deeper horizon.

Although F430 was detected at 88 mbsf, relatively high background and its negative shift nearby F430 prevents from precise quantification of F430. These features would be derived from complex organic matrices in the sample. Especially, the negative shift of the baseline would be caused by ion suppression due to decreasing ionization efficiency of F430 by organic interferences. Co-eluting organic matrix can also cause reduction of coenzyme F430 ionization efficiency. Hence, the affect of organic matrices should be removed to get better chro-
One reason for high background on the chromatogram may be zero-collision energy MRM applied in this study. Applying normal MRM method may be helpful for reduction of interference from organic matrix on the chromatogram. However, it is hard to occur product ions even if high collision energy is applied. In our earlier study, a fragment ion with $m/z = 844.3$ was a most significant product when 66 V of collision energy was applied, but parent ion is still abundant (Fig. 2, Kaneko et al., 2014). Applying normal MRM method ($975.4 \rightarrow 844.3$) sacrifices the sensitivity and does not mean substantial resolution of reduction of ionization efficiency and ion suppression. More fundamental resolution to get a better chromatogram would be a removal of organic matrices before ionization.

Silica gel column chromatography to remove organic matrices

Silica gel column chromatography has been used to classify organic molecules and remove organic matrices in samples. This technique can be widely applicable from nonpolar to polar lipids and porphyrins (tetrapyrrole compounds). Coenzyme F430M is well absorbed on silica gel column and is not eluted by organic solvents with high elution force including dichloromethane, methanol and acetonitrile but elute with 0.1 M NaClO₄ acetonitrile/H₂O (80/20 v/v). Recovery during silica gel chromatography was 71% ± 0.9 ($n = 4$).

Silica gel column chromatography was tested in the F430 fraction extracted from sediment at 88 mbsf off Shimokita Peninsula to remove organic matrices. In this study, an aliquot of the sample was loaded on the silica gel column and separated into three fractions. On the MRM chromatogram of F1 (methanol fraction), F430 was not detected while increased background and negative peaks of ion suppression were appeared (Fig. 3). On the chromatogram of F2 (acetonitrile), F430 as well as increased background and ion suppression were not observed. On the chromatogram of the final fraction F3 (0.1 M NaClO₄ in acetonitrile/H₂O), F430 and its homologues were detected with better peak shape compared with original samples, and high background and negative peaks were no longer detected. Thus, the silica gel chromatography is useful to remove organic matrices. The result of quantification of F430 in F3 is 97.9 fmol.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Sample type</th>
<th>Concentration</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBK</td>
<td>Granules</td>
<td>$3.30 \times 10^7$ fmol/g-wet</td>
<td>Kaneko et al. (2014)</td>
</tr>
<tr>
<td>Anjo E2</td>
<td>Paddy soil</td>
<td>$2.02 \times 10^3$ fmol/g-wet</td>
<td></td>
</tr>
<tr>
<td>Kawatabi</td>
<td>Paddy soil</td>
<td>$8.70 \times 10^2$ fmol/g-wet</td>
<td></td>
</tr>
<tr>
<td>Chikugo CF</td>
<td>Paddy soil</td>
<td>$3.08 \times 10^2$ fmol/g-wet</td>
<td></td>
</tr>
<tr>
<td>Chikugo RSC</td>
<td>Paddy soil</td>
<td>$1.06 \times 10^2$ fmol/g-wet</td>
<td></td>
</tr>
<tr>
<td>Ita-wari</td>
<td>Groundwater</td>
<td>$8.11 \times 10^2$ fmol/g-wet</td>
<td></td>
</tr>
<tr>
<td>Peru 9H1</td>
<td>Marine sediments</td>
<td>$1.92 \times 10^3$ fmol/g-wet</td>
<td></td>
</tr>
<tr>
<td>Shimokita 11-4</td>
<td>Marine sediments</td>
<td>$6.33 \times 10^2$ fmol/g-wet</td>
<td></td>
</tr>
<tr>
<td>Shimokita 8-4</td>
<td>Marine sediments</td>
<td>$5.29 \times 10^2$ fmol/g-wet</td>
<td></td>
</tr>
<tr>
<td>Shimokita 10-4</td>
<td>Marine sediments</td>
<td>$3.13 \times 10^2$ fmol/g-wet</td>
<td></td>
</tr>
<tr>
<td>Nankai 7H7</td>
<td>Marine sediments</td>
<td>$3.14 \times 10^2$ fmol/g-wet</td>
<td></td>
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<tr>
<td>Nankai 29R2</td>
<td>Marine sediments</td>
<td>$2.60 \times 10^2$ fmol/g-wet</td>
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<tr>
<td>Peru 9H1</td>
<td>Marine sediments</td>
<td>$6.33 \times 10^2$ fmol/g-wet</td>
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<tr>
<td>Shimokita 8-4</td>
<td>Marine sediments</td>
<td>$5.29 \times 10^2$ fmol/g-wet</td>
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<td>Shimokita 10-4</td>
<td>Marine sediments</td>
<td>$3.13 \times 10^2$ fmol/g-wet</td>
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<td>Nankai 7H7</td>
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<td>Nankai 29R2</td>
<td>Marine sediments</td>
<td>$2.60 \times 10^2$ fmol/g-wet</td>
<td></td>
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</tbody>
</table>

**Fig. 2. Mass spectrum of F430M when 66 V of collision energy was applied.**

![Mass spectrum of F430M](image-url)
Quantitative analysis of coenzyme F430

Estimating in situ methanogenic biomass and activity

Coenzyme F430 is thermally unstable and finally epimerized to thermodynamically stable form of 12,13-diepi F430 via 13-epi F430. At the thermodynamic equilibrium (100°C), 88% of coenzyme F430 epimerizes to 12,13-diepi F430 and the process occurs in day scale even at room temperature (Diekert et al., 1981; Mayr et al., 2008). Such a rapid deformation coenzyme F430 implies that it does not accumulate in the native form. The native form of coenzyme F430 can be presents as both free form and MCR-bound form. The free form of coenzyme F430, however, is a precursor of MCR-bound F430 and binding of F430 to apo-protein is an irreversible process (Ankel-Fuchs et al., 1984). Residence time of such an intermediate would be short in cells. Even if it is long due to low metabolic activity in marine sediments, coenzyme F430 would be epimerized. Furthermore, protein matrix of MCR moderate nonplanar deformation and epimerization of coenzyme F430 and incorporation of 12,13-diepi F430 and tunes the chemistry of the active site of Ni ions (Mbofana and Zimmer, 2006). Thus, coenzyme F430 detected in marine sediments should represent MCR-binding F430 in living cells when we assume that the rate of epimerization at the conditions of marine sediments is enough fast.

Coenzyme F430 contents in methanogens have been investigated previously in various cultures of methanogens including *Methanobacterium thermoautotrophicum*, *Methanobrevibacter smithii*, *Methanococcus vannielii*, *Methanospirillum hungatii*, and *Methanosarcina barkeri*. The F430 content ranges from 800 nmol g⁻¹ dry cell (M. thermoautotrophicum and M. barkeri) to 227 nmol g⁻¹ dry cell (M. vannielii) with average of 580 ± 240 nmol g⁻¹ dry cell (Diekert et al., 1981). The prokaryotic cell weight is estimated to range from 2 × 10⁻¹³ g in soil to 36 × 10⁻¹⁵ g in marine sediment (Lipp et al., 2008; Whitman et al., 1998). Hence, abundance of the methanogen cell per g-sediment can be estimated from
Table 2. The activities of F430 expressed by culture-based studies

<table>
<thead>
<tr>
<th>μmol/min/μmol F430</th>
<th>Methanogen specimen</th>
<th>Targets</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>17500</td>
<td>M. thermoautotrophicum strain Marburg</td>
<td>Cell suspension</td>
<td>Schönheit et al. (1980)</td>
</tr>
<tr>
<td>7500</td>
<td>M. thermoautotrophicum strain Marburg</td>
<td>Cell suspension</td>
<td>Perski et al. (1981)</td>
</tr>
<tr>
<td>8000</td>
<td>M. thermoautotrophicum strain Marburg</td>
<td>Cell suspension</td>
<td>Perski et al. (1982)</td>
</tr>
<tr>
<td>19</td>
<td>M. thermoautotrophicum strain Marburg</td>
<td>F430M</td>
<td>Jaun and Pfaff (1988)</td>
</tr>
<tr>
<td>150-300</td>
<td>M. thermoautotrophicum strain Marburg</td>
<td>MCR</td>
<td>Ellermann et al. (1988)</td>
</tr>
<tr>
<td>375</td>
<td>M. thermoautotrophicum strain Marburg</td>
<td>MCR</td>
<td>Ellermann et al. (1989)</td>
</tr>
<tr>
<td>15000</td>
<td>M. thermoautotrophicum strain Marburg</td>
<td>MCR</td>
<td>Goubeaud et al. (1997)</td>
</tr>
</tbody>
</table>

The activities of F430 were expressed in a unit of μmol/min/μmol F430. For conversion of the activity from literature data, following parameters were used: MCR = 300 kDa; protein in Methanogen = 50% of dry cell; MCR = 12% of cell protein; 1 mg MCR = 0.0067 μmol F430.

F430 concentration by using the following equation:

$$ n = \frac{C_{F430} \text{ in sample}}{C_{F430} \text{ in culture} \times m_{cell}} \text{ (cells g}^{-1}) $$

where $C_{F430}$ in sample and $C_{F430}$ in culture denote F430 concentrations in sample and culture, respectively and $m_{cell}$ denotes a mean weight of the cell. In fact, estimated cell abundance from F430 concentration seems to well correlate with that estimated by microbiological techniques including cell counting and gene concentration in various environmental samples (paddy field, microbial mat, groundwater (Kaneko et al., 2014).

Cell densities calculated from F430 concentration are $2.7 \times 10^7$ and $4.7 \times 10^8$ cells g-wet$^{-1}$ 69 and 88 mbsf of offshore Shimokita Peninsula while $4.7 \times 10^7$ and $3.9 \times 10^9$ cells g-wet$^{-1}$ at 60 and 275 mbsf of Nankai Trough. A comparison with total prokaryotic cell at Shimokita Peninsula based on a developed cell enumeration technique using hydrofluoric acid treatment and automated fluorescence image analysis (Morono et al., 2009) suggests that methanogen biomass account for about 30 and 10% of total prokaryotic biomass at 69 and 88 mbsf.

The amount of F430 should precisely reflect the amount of MCR because 2 molecules of F430 are individually bounded to two active sites of single MCR. The rate of methanogenesis can also be correlated with concentration of MCR as well as F430 as described by Michaelis-Menten model. The specific activity of MCR (or F430) has been investigated using cell suspension, extracted MCR and F430M to be about 15,000 nmol/min/μmol F430$^{-1}$ (Table 2, Goubeaud et al., 1997; Ellermann et al., 1988, 1989; Jaun and Pfaff, 1988; Perski et al., 1981, 1982; Schönheit et al., 1980). The protocols to estimate methanogen’s biomass and activity described in this study have some limitations. Great care must be taken when the methods are applied to subsurface sediments because of accumulation of debris from past-living organisms. Preservation of biomarkers to detect living prokaryotic signals has been often argued in previous study (e.g., Lipp and Hinrichs, 2009; Schouten et al., 2010; Xie et al., 2014). Intact coenzyme F430 may not accumulate in natural environment as a fossil compound due to its unstable nature (Thauer and Bonacker, 1994; Diekert et al., 1981). Since F430 is readily degraded by both biological and abiological processes after cell death, the degradation rate in the terrestrial environments can be faster than DNA and polar intact lipids. However, the degradation rates of F430 in marine sediments where temperature, oxygen and microbial activities are low have not been investigated. The utility of F430 as a biomarker to investigate modern methanogenesis has to be assessed by degradation experiment of F430 at the conditions of marine sediments in the future study.

In this study, conversion of methanogen’s biomass and activities from F430 was conducted using reference data of culture-based studies. However F430 contents in a cell and its activity in marine sediments will not necessarily be the same as in a culture where substrate is abundant.

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Aoike, K. (2007) CK06-06 D/V Chikyu Shakedown Cruise

required for better estimation of methanogenesis rate degradation rate of F430 in anoxic marine sediments are between F430 concentration and biomass/activities and biomass and activities in marine sediments were quite calculated from F430 concentration however estimated methanogenic biomass and activities

trices for better quantification. Theoretically, methanogenic biomass and activities in situ can be calculated from F430 concentration however estimated biomass and activities in marine sediments were quite higher than those estimated by other microbiological and 14C tracer methods. Further studies to clarify relationship between F430 concentration and biomass/activities and degradation rate of F430 in anoxic marine sediments are required for better estimation of methanogenesis rate in situ.

Acknowledgments—This study was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI Grants 25601666 (to M.K.).

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