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Isotopomeric characterization of nitrous oxide produced by reaction of enzymes extracted from nitrifying and denitrifying bacteria

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Abstract. Nitrous oxide (N_2O) is a potent greenhouse gas and produced in denitrification and nitrification by various microorganisms. Site preference (SP) of ^{15}N in N_2O, which is defined as the difference in the natural abundance of isotopomers $^{14}N^{15}NO$ and $^{15}N^{14}NO$ relative to $^{14}N^{14}NO$, has been reported to be a useful tool to quantitatively distinguish N_2O production pathways. To determine representative SP values for each microbial process, we firstly measured SP of N_2O produced in the enzyme reaction of hydroxylamine oxidoreductase (HAO) purified from two species of ammonia oxidizing bacteria (AOB), *Nitrosomonas europaea* and *Nitrosococcus oceani*, and that of nitric oxide reductase (NOR) from *Paracoccus denitrificans*. The SP value for NOR reaction (-5.9 ± 2.1 ‰) showed nearly the same value as that reported for N_2O produced by *P. denitrificans* in pure culture. In contrast, SP value for HAO reaction (36.3 ± 2.3 ‰) was a little higher than the values reported for N_2O produced by AOB in aerobic pure culture. Using the SP values obtained by HAO and NOR reactions, we calculated relative contribution of the nitrite (NO_2^-) reduction (which is followed by NO reduction) to N_2O production by *N. oceani* incubated under different O_2 availability. Our calculations revealed that previous in vivo studies might have underestimated the SP value for the NH_2OH oxidation pathway possibly due to a small contribution of NO_2^- reduction pathway. Further evaluation of isotopomer signatures of N_2O using common enzymes of other processes related to N_2O would improve the isotopomer analysis of N_2O in various environments.

1 Introduction

Nitrous oxide (N_2O) is a potent greenhouse gas and contributes indirectly to the destruction of the ozone layer in the stratosphere (Ravishankara et al., 2009). Production of N_2O on earth is mainly controlled by microbial processes that include nitrification and denitrification (Stein and Yung, 2003). In nitrification, autotrophic microorganisms like ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) oxidize ammonia to nitrite (NO_2^-) and produce N_2O as a byproduct under mainly aerobic conditions (Casciotti et al., 2011). However, various heterotrophic denitrifying microorganisms such as archaea, bacteria, and fungi reduce nitrate (NO_3^-) or NO_2^- to dinitrogen (N_2) as electron acceptors for anaerobic respiration and produce N_2O as an intermediate under mainly anaerobic conditions (Hayastu et al., 2008). Thus, production and consumption of N_2O consist of many microbiological functions, including

autotrophic/heterotrophic and oxic/anoxic processes. Investigation of N_2O production pathways is useful to understand the nitrogen cycle and relevant microorganisms in various environments.

Natural abundance ratios of isotopomers, a set of molecules containing various stable isotopes, have been used to analyze the N_2O production pathways, and several studies have reported isotopomer ratios of each N_2O production pathway using several strains belonging to bacteria, archaea, and fungi (Casciotti et al., 2011; Frame and Casciotti, 2010; Santoro et al., 2011; Sutka et al., 2008, 2006, 2004; Toyoda et al., 2005; Yoshida, 1988). These studies showed that the site preference (SP), which was defined as the difference in ^{15}N enrichment between the center (α) and the terminal (β) N atoms in N_2O molecules, is a powerful tool to quantitatively distinguish the production pathways such as bacterial NO_2^- reduction and bacterial hydroxylamine (NH_2OH) oxidation, as the SP value for each production pathway was found to be independent of concentrations or isotope ratios of substrates (Toyoda et al., 2005; Sutka et al., 2003, 2004, 2006). To date, isotope analysis has been applied to N_2O produced in various ecosystems, including natural and agricultural soils, oceans, rivers, wastewater treatment plants (Goldberg et al., 2010; Koba et al., 2009; Park et al., 2011; Sasaki et al., 2011; Toyoda et al., 2011; Well et al., 2008; Yoshida and Toyoda, 2000). However, as argued by Baggs et al. (2008), the isotopomer signatures were reported for only a few bacterial strains and the variation of the signatures among various species was not fully evaluated. In addition, previous reports on the SP value of N_2O produced by NH_2OH oxidation might have been biased by simultaneous N_2O production by NO_2^- reduction, since some AOB have denitrifying enzymes and produce N_2O by NO_2^- reduction in addition to NH_2OH oxidation (Arp et al., 2003). In consideration of this problem, Frame and Casciotti (2010) estimated the SP value of N_2O produced by NH_2OH oxidation using the relationship between SP and oxygen isotope ratios in N_2O obtained in incubation experiments with *Nitrosomonas marina* C-113a, a marine ammonia-oxidizing bacterium, under various oxygen concentrations. Clearly, more information on SP produced by other strains should be obtained for the better use of SP and precise estimation of the contribution from each N_2O production pathway.

In this study, we report the isotopomer ratios of N_2O produced in vitro using enzymes extracted and purified from two strains of AOB (*Nitrosococcus oceani* and *Nitrosomonas europaea*) and one species of denitrifying bacteria (*Paracoccus denitrificans*). Our experiments have the advantage that isotope effects related to the N_2O production by enzymes (hydroxylamine oxidoreductase (HAO) in AOB and nitric oxide reductase (NOR) in denitrifier) can be directly determined and that the effects related to other processes such as diffusion of substrate/product through cell membrane and reactions mediated by other enzymes can be excluded. We also measure isotopomer ratios of N_2O produced in vivo by *N. oceani* under different oxygen concentrations and estimate the relative contribution of NH_2OH oxidation and NO_2^- reduction using the isotopomer signatures obtained in vitro.

2 Materials and methods

2.1 Cultivation of the bacterial strains

Nitrosococcus oceani strain NS58 was kindly supplied by Dr. H. Urakawa (Florida Gulf Coast University). Phylogenetic and morphological analyses indicated a close systematic relationship of the bacterium with *N. oceani* ATCC19707 (Hozuki et al., 2010). The bacterium was cultivated in the $(NH_4)_2SO_4$-supplemented artificial seawater (37.8 mM NH_4^+), of which the pH was buffered to 7.8 by 50 mM MOPS (3-morpholinopropanesulfonic acid) as described in detail in a previous report (Hozuki et al., 2010). The inoculated medium in the conical flask was shaken reciprocally at 120 rpm at 25 °C in a dark condition. After cultivation for 7–10 days and the concentration of NO_2^- in the medium had reached 25–30 mM, the culture of the bacterium in the late-log or stationary phase was used for the in vivo experiments as described below. Large-scale cultivation of the bacterium to prepare the starting material for purification of HAO was carried out with the same procedure, but using a glass bottle 10 L in volume with vigorous air ventilation through a sterilized air filter.

Cultivated cells of *Nitrosomonas europaea* ATCC19718, which was obtained by large-scale cultivation in accordance to a previous study (Yamanaka and Shinra, 1974), were kindly supplied by Dr. Y. Fukumori (Graduate School of Natural Science and Technology, Kanazawa University) and were used as a starting material for the purification of HAO. *Paracoccus denitrificans* ATCC35512 was cultured under denitrifying conditions and used for preparing a purified NOR according to the previous report by Fujiwara and Fukumori (1996).

2.2 Purification of hydroxylamine oxidoreductase (HAO)

HAO was purified from the cultivated NS58 cells through three preparative steps, including $(NH_4)_2SO_4$ fractionation, gel-filtration and hydrogen-bonding chromatography according to a previous report (Hozuki et al., 2010). Catalytic activity of HAO was analyzed by spectrophotometrical measurement of the NH_2OH-dependent reduction of potassium ferricyanide as reported previously (Hozuki et al., 2010). Briefly, the purified enzyme was mixed with 1 mL of the reaction solution containing 0.1 M sodium phosphate buffer (pH 7.8), 20 µM NH_2OH and 100 µM potassium ferricyanide. Then, the decreasing rate of absorbance at 420 nm of the solution was monitored using a cuvette with an optical path length of 1cm and a spectrophotometer (MPS-2000, Shimadzu, Kyoto,

Japan). The difference in the millimolar extinction coefficient ($\Delta\varepsilon_{mM}$) at 420 nm between ferricyanide and ferrocyanide was 1.02 $mM^{-1}\,cm^{-1}$. We obtained 1.8 mg of the purified HAO with enzymatic activity of 37 unit mg $protein^{-1}$ (1 unit is equivalent to the activity where 1 µmol of NH_2OH is oxidized in a minute) and used it for the assay of N_2O-generating activity. HAO was also purified from *N. europaea* according to the method of Yamanaka et al. (1979) with some modifications. We found lower catalytic activity for the enzyme purified from *N. europaea* (6.7 unit mg $protein^{-1}$); this is probably because we used an old stock of the cultivated bacterial cells which had been kept for about 10 years at −30 °C.

2.3 Quantitative and isotopomeric analysis of N_2O produced during oxidation of NH_2OH with HAO

In a 69 mL glass vial (Maruemu Corp., Osaka, Japan), 10 mL of substrate solution was prepared so that it contains 0.1–3 mM hydroxylamine, 1 mM potassium ferricyanide as an electron acceptor, and 10 mM sodium phosphate as buffer (pH = 7.8). After the vial was sealed with a butyl rubber stopper and an aluminum cap, air in the headspace was replaced with pure N_2 (Shizuoka Sanso Co., Shizuoka, Japan), and then the reaction was started by injecting 0.1 unit of the HAO extracted from *N. oceani* or *N. europaea*. Experiments with 3 mM NH_2OH were conducted on four different dates (A–D). In experiments C and D, the lot number of NH_2OH reagent (hydroxylamine hydrochloride, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was different from that used in experiments A and B. After incubating the vial for 2 h at 25 °C or for 12 h on ice, gas samples were extracted and analyzed as described below. Concentration of NO_2^- was determined spectrophotometrically by a diazo-coupling method (Nicholas and Mason 1957).

2.4 Purification of nitric oxide reductase (NOR) from *Paracoccus denitrificans*

NorBC-type NO reductase was purified from the cultivated *P. denitrificans* cells according to a previous report (Fujiwara and Fukumori 1996) with some modification. The membrane fraction was prepared from the bacterial cells that had been cultivated anaerobically in the presence of NO_3^-. NO reductase was extracted from the membrane fraction by treating with the 1 % (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Dojindo Lab., Kumamoto, Japan). It was then fractionated by anion-exchange chromatography using DEAE-Toyopearl 650 M gel (Tosoh, Tokyo, Japan).

NO-reducing activity of the purified enzyme was measured spectrometrically by monitoring NO-dependent oxidation of horse ferrocytochrome *c* (Nacalai Tesque, Kyoto, Japan). Experimental procedure was detailed in a previous report (Fujiwara and Fukumori 1996). Before starting the assay of NO-reducing activity, dissolved oxygen in the solution was enzymatically removed using D-glucose oxidase/catalase system in order to prevent its reaction with NO. A stock solution of NO-saturated ethanol (11.9 mM) was prepared by purging ethanol with pure NO gas (Sumitomo Seika Chemicals Co., Ltd. Osaka, Japan) (Seidell and Linke, 1965). Oxidation rate of ferrocytochrome *c* was measured by monitoring the decrease in the absorbance at 550 nm with a spectrophotometer (MPS-2000, Shimadzu, Kyoto, Japan), whereby the difference in millimolar extinction coefficient ($\Delta\varepsilon_{mM}$, 21.0 $mM^{-1}\,cm^{-1}$) at 550 nm between the reduced and oxidized form of horse ferro- and ferricytochrome *c* was used. NO-reducing activity of the purified enzyme was estimated to be 35 unit mg $protein^{-1}$ (1 unit is equivalent to the activity where 1 µmol of NO is reduced in a minute).

2.5 Isotopomeric analysis of N_2O produced by enzymatic reduction of NO

In the same glass vial that had been used for NH_2OH oxidation experiments, a 10 mL solution was prepared so that it contained 10 mM sodium phosphate buffer (pH 6.0), 2.9 µM horse cytochrome *c*, 1.0 mM sodium ascorbate and 0.1 mM N, N, N′, N′-tetramethyl-p-phenylenediamine (TMPD). The reaction was started by injecting the NO-saturated ethanol to yield final concentration of 50 µM, and the purified NO reductase (0.005 unit) by using a gastight syringe (VICI Precision Sampling Inc., Baton Rouge, LA). Experiments were conducted on three different dates (A, C, and D). After incubating the vial for 2 h at 25 °C or for 12 h on ice, the gas phase in the headspace of the reaction vial was extracted and the isotopomeric analysis of the produced N_2O was conducted as mentioned below.

2.6 Quantitative and isotopomeric analysis of N_2O produced from cultivated *N. oceani* cells

The 70 mL culture of the *N. oceani* NS58 was centrifuged at 9800 g for 60 min at 4 °C (refrigerated centrifugator model 3700, Kubota Corp., Tokyo, Japan). Obtained pelleted cells were suspended in the 70 mL of the pH-buffered and $(NH_4)_2SO_4$-supplemented artificial seawater (see Sect. 2.1), then were incubated with gentle stirring at 25 °C in dark for 30 min to remove NO_2^- that accumulate in the medium during the cultivation. The resulting cell suspension was centrifuged again under the same condition, and the cell pellet was resuspended in the same volume of the freshly prepared culture medium and used as the washed cell suspension.

Reaction solution was prepared in a glass vial by mixing 1.0 mL of the washed cell suspension and 9.0 mL of the cultivation medium. Cell density of the medium in the reaction vial was estimated at 5.8×10^6 cells mL^{-1} by direct counting of the fixed and 4′,6-diamidino-2-phenylindole (DAPI)-stained cells using an epifluorescent microscope according to

Takenaka et al. (2007). After sealing, the headspace (59 mL in volume) of the vial was replaced with a pure N_2 or O_2/N_2 mixture that contained 2 % (v/v) O_2 (Shizuoka Sanso Co., Shizuoka, Japan) by gently bubbling for 15 min. In addition to the anaerobic (0 % O_2) and the microaerobic (2 % O_2) vials, an aerobic vial without gas replacement was prepared. Incubation of the bacterial cells in the vials was carried out by gentle shaking at 25 °C in dark. After 24 or 48 h from starting the incubation, 50 µM of 10 M NaOH solution was added into the vials to stop the microbial reaction. Quantification and measurement of isotopomer ratios of N_2O gas released into the headspace of the incubation vial were performed as described below. Concentration of accumulated NO_2^- was also measured as described above.

2.7 Measurement of N_2O concentration and isotopomer ratios

Concentrations and isotopomer ratios of N_2O were measured using an online analytical system that originally was developed for N_2O dissolved in water samples (Toyoda et al., 2002). The system consists of a gas extraction chamber with a septum for syringe injection, traps made of stainless steel tubing or glass, a gas chromatograph (Agilent 6890, Agilent Technologies Japan, Ltd., Tokyo) and an isotope-ratio monitoring mass spectrometer (MAT 252, ThermoFisher Scientific KK, Yokohama) equipped with GC interface.

Using a gastight syringe, 0.1 to 1.5 mL of the gas was extracted from the headspace of the sample vial and was injected into the gas extraction chamber. The sample gas was then transferred by He carrier gas to chemical traps ($Mg(ClO_4)_2$ and NaOH on support) to remove H_2O and CO_2, and N_2O was concentrated on glass beads packed in a U-shaped trap at liquid N_2 temperature. After further purification on GC, N_2O was introduced into the mass spectrometer for isotope ratio monitoring. Site-specific nitrogen isotope analysis was conducted with ion detectors modified for mass analysis of fragment ions of N_2O (NO^+) that contain an N atom in the center position of the N_2O molecules (Toyoda and Yoshida, 1999). Concentration and bulk nitrogen and oxygen isotope ratio of N_2O was determined by analysis of N_2O molecule ions (N_2O^+). Pure N_2O was used as a reference gas for isotopomer ratios. Notation of isotopomer ratios of N_2O is shown below.

$$\delta^{15}N^{i}_{sample} = {}^{15}R^{i}_{sample} / {}^{15}R_{standard} - 1 \quad (1)$$

$$\delta^{18}O_{sample} = {}^{18}R_{sample} / {}^{18}R_{standard} - 1 \quad (2)$$

In Eqs. (1) and (2), ^{15}R and ^{18}R represent $^{15}N/^{14}N$ ratio and $^{18}O/^{16}O$ ratio, respectively. Subscript "sample" and "standard" indicate isotope ratios for sample and the standard (atmospheric N_2 for nitrogen and Vienna Standard Mean Ocean Water (VSMOW) for oxygen), respectively. Superscript i is α, β, or bulk, which respectively designates central, peripheral, or average isotope ratios in nitrogen atom(s) in N_2O molecules. We also define the ^{15}N site preference (SP) as an illustrative parameter of intramolecular distribution of ^{15}N (Yoshida and Toyoda, 1999). The precision of measurement is better than 0.5 ‰ for $\delta^{15}N^{bulk}$ and $\delta^{18}O$, and better than 1.0 ‰ for $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$.

$$^{15}N - \text{Site preference (SP)} = \delta^{15}N^{\alpha} - \delta^{15}N^{\beta} \quad (3)$$

The $\delta^{15}N$ of NH_2OH was measured using an elemental analyzer coupled with isotope ratio mass spectrometer. Statistical analysis was performed using Excel 2011 (Microsoft, USA). The statistical difference was determined by a two-sided Student's t test. A difference of $p < 0.05$ was considered significant.

3 Results

3.1 Concentrations and isotopomer ratios of N_2O produced during oxidation of NH_2OH with HAO

The purity of HAO obtained from *N. oceani* NS58 was confirmed by the SDS-PAGE technique. As shown in Supplement Fig. S1, SDS-PAGE results of the purified sample revealed a single protein band with a molecular weight of approximately 182 000, following pretreatment with SDS (Lane 2). Upon treatment of the enzyme with both SDS and β-mercaptoethanol, the major band accompanied by minor protein bands with molecular weights of 140 000 and 60 000 appeared on the gel (Lane 1). The chemical cleavage of the thioester bond between heme c and a Cys residue in HAO resulted in the appearance of a protein band of molecular weight 60 000 (Lane 3), suggesting that the enzyme is composed of a homotrimer of subunit proteins covalently linked together and mediated by prosthetic heme c molecules, as previously reported for *N. europaea* HAO (Terry and Hooper, 1981; Igarashi et al., 1997). The two minor protein bands observed in Lane 1 possibly correspond to the monomeric and dimeric configurations of the HAO subunit. In general, AOB possesses two types of hydroxylamine-oxidizing enzymes: HAO, with a trimeric configuration of the octaheme c subunit and cytochrome P460, a monoheme protein with a molecular weight of only approximately 18 000 (Numata et al. 1990). Gel filtration was used for purification of *N. oceani* NS58 HAO; thus, contamination of the cytochrome P460 in the enzyme preparation should be improbable. Results of electrophoretic analysis are consistent with the absence of components that may interfere with the correct interpretation of our experimental findings (Lanes 1 and 2, Fig. S1).

Figure 1 shows the amount of NO_2^- and N_2O produced during the reaction catalyzed by HAO from *N. oceani* with different initial concentration of NH_2OH (0.1–3 mM). About 5.1–29.0 % of NH_2OH was converted to NO_2^- or N_2O after the reaction, and the ratio of produced NO_2^- and N_2O decreased with initial NH_2OH concentration. Production of N_2O in the blank runs with 1 and 3 mM NH_2OH were 5.9

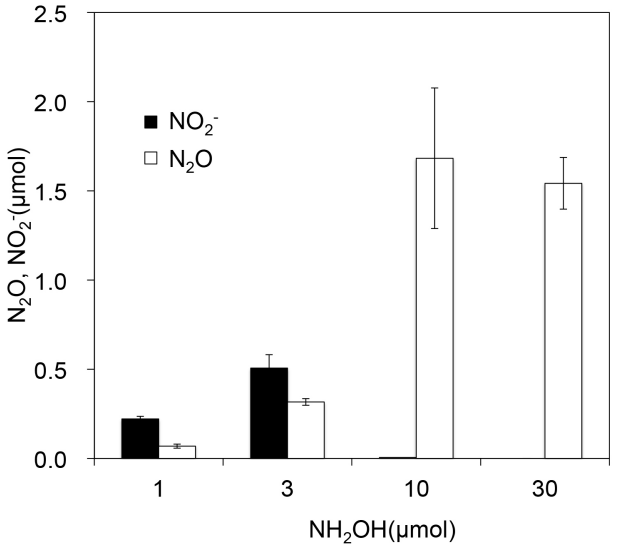

Figure 1. Amounts of N_2O and NO_2^- produced by HAO extracted from *N. oceani* under different substrate (NH_2OH) concentration (expressed as amount in the reaction vessel). The error bar indicates standard deviation for three replicates.

and 7.1 % of that in the presence of HAO (HAO+), respectively (Table 1). With lower NH_2OH concentrations of 0.1 and 0.3 mM, however, production of N_2O in the blank runs was 32.2 and 179.1 % of that observed in the HAO+ runs, respectively (Table 1). For this reason, further experiments were conducted at an initial NH_2OH concentration of 3 mM to examine repeatability of the reaction and effect of bacterial strain and N isotope ratio of NH_2OH. The $\delta^{15}N^{bulk}$ and $\delta^{18}O$ of N_2O showed lower values at high (3 mM) NH_2OH concentration compared to low (1 mM) concentration (Table 1). SP value was independent of substrate concentration or the degree of the reaction, since the difference in SP values between experiments with 1 and 3 mM NH_2OH was insignificant ($p > 0.05$).

Results obtained with an initial NH_2OH concentration of 3 mM showed that $\delta^{15}N^{bulk}$ of N_2O produced by *N. oceani* (experiments A and B) and *N. europaea* (experiment A) was almost constant and about 10 ‰ lower than $\delta^{15}N$ of NH_2OH (−7.0 ‰) (Table 2). Similar $\delta^{15}N$ difference (9 ‰) between N_2O and NH_2OH was observed in experiment D, in which NH_2OH with lower $\delta^{15}N$ value (−43.9 ‰) was used. In experiment C of both *N. europaea* and *N. oceani*, however, $\delta^{15}N$ of N_2O was higher than that of NH_2OH by 1.8–9.9 ‰ (Table 2). The $\delta^{18}O$ also showed a variation among experiments A–D of *N. oceani*, although it is not clear whether the $\delta^{18}O$ of NH_2OH was different between experiments A/B and C/D. When we compare the results from experiment A, the $\delta^{15}N^{bulk}$ and $\delta^{18}O$ of N_2O were not significantly different between the two strains.

In contrast, the SP value of N_2O produced by NH_2OH oxidation with HAO was independent of initial concentration and $\delta^{15}N$ value of NH_2OH or degree of reaction process (Tables 1 and 2). Moreover, the difference in SP between experiments with HAO extracted from *N. oceani* in experiments A, B , C and D (average: 36.2 ± 1.7 ‰, $n = 7$) and *N. europaea* in experiments A and C (average: 36.6 ± 3.3 ‰, $n = 4$) was insignificant ($p > 0.05$).

3.2 Concentration and isotopomer ratios of N_2O produced during reduction of NO with NOR

Table 3 shows concentration and isotopomer ratios of N_2O produced by the reduction of NO with NOR. About 65.0–149.2 % of NO was converted to N_2O. The concentration of N_2O was 17 times and 7.3 times higher in the presence of NOR (NOR+) compared to the blank runs without NOR. SP values of N_2O from the enzymatic reaction showed little variation (-5.9 ± 2.1 ‰) and were lower than SP values observed in the control runs (15.1–16.8 ‰) ($p < 0.05$).

3.3 Isotopomer ratio of N_2O produced by *N. oceani* under different initial O_2 concentration

The amount of N_2O produced by *N. oceani* was 2.9 ± 0.9 ($n = 6$), 8.9 ± 0.5 ($n = 2$), and 18.1 ± 3.6 ($n = 5$) nmol for O_2 concentrations of 0, 2, and 21 %, respectively. Production of both N_2O and NO_2^- was much higher under aerobic conditions than under anaerobic conditions ($p < 0.05$, Fig. 2). Isotopomer ratios of N_2O showed a strong dependence on initial O_2 concentration (Fig. 3). They were more enriched under aerobic conditions compared to anaerobic conditions, and positive correlation between the isotopomer ratios was observed (Fig. 3, $R^2 = 0.89$, $p < 0.05$).

4 Discussion

The characteristic SP value of N_2O produced during the in vitro oxidation of NH_2OH with HAO from the two strains of AOB (36.3 ± 2.3 ‰) indicates that this parameter is determined by the enzymatic reaction step and is not affected by other factors such as concentration, the extent of reaction, and nitrogen isotope ratios of substrate (Table 2). This study demonstrates the first direct evidence of isotopomeric fractionation of N_2O produced by enzymatic reactions, confirming the robustness of SP of N_2O as predicted by previous studies (e.g., Frame and Casciotti. 2010).

Observed dependence of product NO_2^- / N_2O ratio on initial NH_2OH concentration could be caused by the availability of electron acceptors. The oxidation of NH_2OH with HAO mainly provides NO_2^- as a product when enough electron acceptors are supplied, while the reaction is likely to produce N_2O when the amount of electron acceptors is not enough to complete the reaction as proposed by Yamanaka and Sakano (1980). In the present study, electron acceptors (potassium

Table 1. Concentration and isotopomer ratios of N_2O produced during NH_2OH oxidation by HAO enzyme extracted from *N. oceani* with different concentrations of NH_2OH.

HAO	NH_2OH		Concentration ($\mu mol\,L^{-1}$)	Yield (%)	$\delta^{15}N^{bulk}$ (‰)	$\delta^{15}N^{\alpha}$ (‰)	$\delta^{15}N^{\beta}$ (‰)	$\delta^{18}O$ (‰)	SP (‰)
+	3 mM	Average ± SD	23.9 ± 2.2	5.1 ± 0.4	−17.9 ± 0.2	0.1 ± 0.2	−36.0 ± 0.2	37.8 ± 0.1	36.1 ± 0.2
Blank	3 mM		1.7	0.4	−17.0	−2.9	−31.1	32.3	28.2
+	1 mM	Average ± SD	26.1 ± 6.1	16.8 ± 3.9	−13.4 ± 0.2	4.7 ± 0.4	−31.6 ± 0.3	42.6 ± 0.2	36.3 ± 0.6
Blank	1 mM		1.5	1.0	−15.4	1.0	−31.8	37.7	32.8
+	0.3 mM	Average ± SD	4.9 ± 0.3	10.6 ± 0.3	−5.8 ± 0.2	12.0 ± 0.3	−23.6 ± 0.2	44.5 ± 0.1	35.6 ± 0.3
Blank	0.3 mM		1.6	3.4	−15.1	2.9	−33.1	47.7	36.0
+	0.1 mM	Average ± SD	1.1 ± 0.2	6.9 ± 1.2	−10.7 ± 0.5	6.6 ± 0.4	−28.1 ± 0.4	45.1 ± 0.8	34.7 ± 0.9
Blank	0.1 mM		1.9	12.3	−15.5	0.5	−31.5	50.4	32.0

Concentration is that in the gas phase (head space) in the vial. N_2O yield represents the fraction of N atoms converted to N_2O from substrate NH_2OH ($[N_2O\text{-}N]/[NH_2OH\text{-}N]$). Isotopomer ratios for "HAO+" were corrected for the blank. Average and SD (standard deviation) were calculated for samples, including HAO in each condition ($n = 3$).

Table 2. Concentration and isotopomer ratios of N_2O produced during NH_2OH oxidation by HAO enzyme extracted from *Nitrosococcus oceani* and *Nitrosomonas europaea* with 3 mM of NH_2OH.

Experiment	HAO	Concentration ($\mu mol\,L^{-1}$)	Yield (%)	$\delta^{15}N^{bulk}$ (‰)	$\delta^{15}N^{\alpha}$ (‰)	$\delta^{15}N^{\beta}$ (‰)	$\delta^{18}O$ (‰)	SP (‰)
Nitrosococcus oceani (HAO)								
A	+	31.0	13.3	−16.5	2.1	−35.1	41.6	37.1
A	+	28.1	12.1	−17.1	1.8	−36.0	41.3	37.8
Average		29.6 ± 2.1	12.7 ± 0.9	−16.8 ± 0.4	1.9 ± 0.2	−35.5 ± 0.6	41.5 ± 0.2	37.4 ± 0.5
A	Blank	4.7	2.0	−14.8	−4.0	−25.6	36.3	21.6
B	+	25.0	10.7	−17.7	0.0	−35.4	37.2	35.3
B	+	21.4	9.2	−18.0	0.0	−16.4	37.6	35.8
B	+	25.5	10.9	−18.1	−0.2	−16.5	37.3	35.5
Average		23.9 ± 2.2	10.3 ± 1.0	−17.9 ± 0.2	−0.1 ± 0.1	−35.7 ± 0.2	37.4 ± 0.2	35.6 ± 0.3
B	Blank	1.7	0.7	−17.0	−2.9	−31.1	32.3	28.2
C	+	38.7	16.6	−42.1	−25.4	−58.8	33.9	33.3
C	Blank	0.6	0.3	−39.7	−24.2	−55.2	33.1	31.1
D	+	24.7	10.6	−52.7	−33.5	−71.9	56.8	38.4
D	Blank	2.6	1.1	−49.4	−38.2	−60.5	54.7	22.3
D	Blank	0.1	0.03	−46.2	−27.0	−65.5	55.3	38.5
Nitrosomonas europaea (HAO)								
A	+	32.8	14.1	−14.4	4.9	−33.7	42.5	38.6
A	+	36.4	15.6	−13.8	4.6	−32.2	42.9	36.8
A	+	34.7	14.9	−17.1	2.4	−36.5	42.8	38.9
Average		34.6 ± 1.8	14.9 ± 0.9	−15.0 ± 1.5	2.9 ± 1.2	−33.0 ± 1.9	41.8 ± 0.2	35.9 ± 0.9
A	Blank	4.7	2.0	−14.8	−4.0	−25.6	36.3	21.6
C	+	5.0	2.2	−34.0	−18.1	−49.9	37.4	31.8
C	Blank	0.3	0.1	−39.7	−24.2	−55.2	33.1	31.1

Experiments A, B, C and D were conducted on different dates. The initial $\delta^{15}N$ value of NH_2OH was −7.0 ‰ for A and B, −43.9 ‰ for C and D. Concentration is that in the gas phase (head space) in the vial. N_2O yield represents the fraction of N atoms converted to N_2O from substrate NH_2OH ($[N_2O\text{-}N]/[NH_2OH\text{-}N]$). Isotopomer ratios for "HAO+" were corrected for the blank. Average and standard deviation were calculated for samples, including HAO.

ferricyanide) might have been depleted under conditions of 1 and 3 mM NH_2OH because its initial concentration was kept constant. With lower NH_2OH concentrations (0.1 and 0.3 mM), the production rate of N_2O was smaller than that of NO_2^-, and the amount of produced N_2O became closer to that produced in the blank experiments. The amount of blank N_2O was almost constant (70–90 nmol; note that the "yield" listed in Table 1 shows the amount of N_2O-N relative

Table 3. Concentration and isotopomer ratios of N_2O during NO reduction by NOR extracted from *Paracoccus denitrificans*.

	NOR	Concentration ($\mu mol\,L^{-1}$)	Yield (%)	$^{15}N^{bulk}$ (‰)	$^{15}N^{\alpha}$ (‰)	$^{15}N^{\beta}$ (‰)	^{18}O (‰)	SP (‰)
A	+	5.1	129.4	17.1	14.3	19.9	24.0	−5.7
A	+	5.0	127.9	17.1	15.1	19.1	24.1	−4.0
A	+	5.9	149.2	17.1	14.6	19.6	22.9	−5.0
A	Blank	0.3	7.6	14.5	22.0	6.9	−7.5	15.1
C	+	2.6	65.0	11.0	6.5	15.5	18.7	−9.0
C	Blank	0.3	7.4	11.7	19.9	3.5	13.5	16.4
D	Blank	0.4	8.9	16.3	24.7	8.0	−6.5	16.8
Average		4.6 ± 1.4	117.9 ± 36.5	15.6 ± 3.0	12.6 ± 4.1	18.5 ± 2.1	22.4 ± 2.5	-5.9 ± 2.1

Experiments A, C and D were conducted on different dates. Concentration is that in the gas phase (head space) in the vial. N_2O yield represents the fraction of N atoms converted to N_2O from substrate NO ([N_2O-N / NO-N]). Isotopomer ratios for NOR+ were corrected for the blank and were averaged for each experiment. SD represents standard deviation for each experiment. For experiment C, results of experiment D were adopted as the blank, because the blank $\delta^{18}O$ value obtained in experiment C was significantly different from that obtained in experiments A and D.

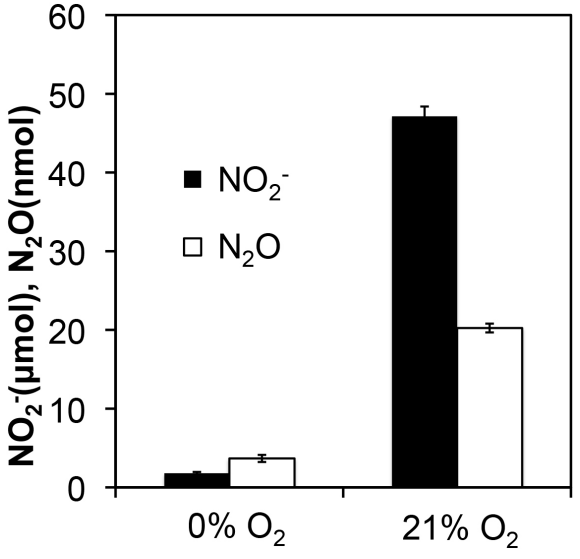

Figure 2. Amount of NO_2^- and N_2O produced from 378 µmol of NH_4^+ in concentrated cell suspensions of *N. oceani* under different initial O_2 concentrations ($n = 2$).

to initial NH_2OH), and it must have been produced by non-catalytic reactions.

The yield of N_2O produced by the reduction of NO with NOR exceeded 100 %, which could be caused by underestimation of initial NO concentration. Since the NO-saturated ethanol solution was prepared by purging the ethanol with pure NO gas in a sealed vial at room temperature (25 °C), we calculated the NO concentration based on NO solubility at 25 °C and 1 atm. However, there is a possibility that the vial inside was pressurized and the excess NO dissolved in ethanol during the preservation at 4 °C.

The SP value of N_2O produced during the in vitro reduction of NO with NOR from *P. denitirifcans* (-5.9 ± 2.1 ‰) agrees with the value reported for N_2O produced in vivo from NO_3^- reduction by the same species (-5.1 ± 1.8 ‰, Toyoda et al. 2005). Therefore, our results prove that the factor-controlling SP value of N_2O produced in NO_3^- reduction is the reaction with NOR rather than other reaction steps, including diffusion of substrate and product through cell membranes. However, the SP value is slightly lower than SP values reported for N_2O produced from NO_3^- or NO_2^- reduction by other denitrifying bacteria or some species of AOB (−0.8 to +0.1 ‰) (Sutka et al., 2004, 2006), and slightly higher than the value estimated for N_2O produced from NO_2^- reduction by *N. marina 113a*, oceanic AOB (-10.7 ± 2.9 ‰; Frame and Casciotti 2010). This implies that SP might depend on a small structural difference in the active center of NOR between the studied species, or that previously reported SP for N_2O from NO_3^- / NO_2^- reduction could have been affected by other processes like NH_2OH oxidation.

It is known that AOB produce N_2O as a byproduct during the oxidation of NH_2OH to NO_2^- with HAO and that some species of AOB can also produce N_2O from nitrite by the sequential reduction of nitrite to NO (using NIR) followed by reduction of NO (using NOR) (Klotz et al., 2006; Arp et al., 2003). The latter pathway is often referred to as nitrifier-denitrification (Wrage et al., 2001) and is believed to occur under anaerobic conditions. Although elucidating the reason why those AOB function as nitrifier-denitrification requires study of a wider spectrum of strains, detoxification of accumulated nitrite, co-respiration of oxygen and nitrite, and other mechanisms have been proposed (Beaumont et al., 2004; Stein, 2011).

In our bacterial culture experiments, production of both NO_2^- and N_2O are enhanced under aerobic conditions (Fig. 2) because O_2 is required for ammonium oxidation, the first step of the successive reaction to NO_2^-. Although we did not measure O_2 concentration during the aerobic incubation (21 % O_2), oxygen concentration must have been

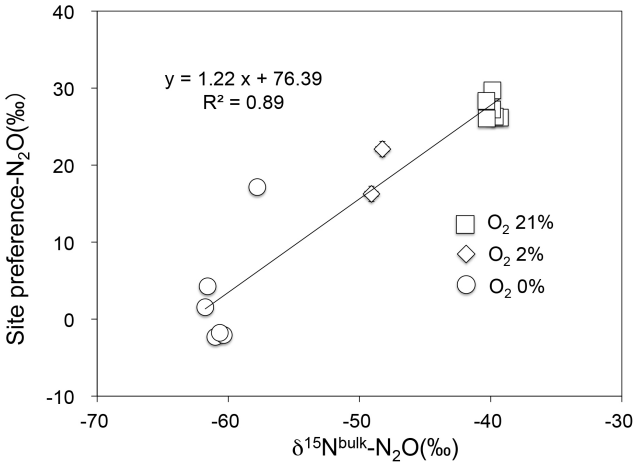

Figure 3. Site preference and δ^{15}N of N_2O in concentrated cell suspensions of *N. oceani* under different initial O_2 concentrations. Error bar indicates the measurement error.

kept high enough to maintain the aerobic condition for the following reasons: (1) it is reported that the measured ratio of consumed O_2 and produced NO_2^- during aerobic incubation of *N. oceani* (1.35–1.43) is consistent with the ratio calculated from the stoichiometry of ammonia oxidation to nitrite (1.50) (Gundersen, 1966); (2) oxygen consumption by respiration using endogenous organics is low (Gundersen, 1966); and (3) a quantitative estimate based on the stoichiometry and the amount of the product (NO_2^- and N_2O) in this study indicates that the oxygen consumption was relatively small (e.g., oxygen concentration should have decreased from 21 % to ca. 18 %).

Furthermore, observed co-variation of SP values of N_2O and oxygen concentrations (Fig. 3) implies that relative contributions from the two production pathways are sensitive to oxygen availability. It has been shown that SP of N_2O produced by nitrifier-denitrification and by NH_2OH oxidation are approximately 0 and 30 ‰, respectively (Sutka et al., 2006). Consequently, the lower SP values produced during nitrification in this study are most likely the result of the addition of N_2O from nitrifier-denitrification. It turns out that NH_2OH oxidation is the dominant N_2O production pathway under aerobic conditions, while NO_2^- reduction is dominant under anaerobic conditions (Fig. 3). We note that even in aerobic conditions (21 % O_2), the SP value of N_2O produced by *N. oceani* is lower than that of N_2O produced by in vitro NH_2OH oxidation with HAO extracted from the same bacteria (Fig. 3). This suggests the NO_2^- reduction pathway is not negligible under aerobic conditions and thus previous studies based on pure culture incubation of AOB underestimated the SP value for N_2O from NH_2OH oxidation.

We further show quantitative estimation of the contribution of NO_2^- reduction to N_2O production. Here we assume that only NH_2OH oxidation and nitrifier-denitrification were the N_2O production pathways, although there was a possibility that N_2O was produced by other unknown processes. This assumption allows a simple interpretation of the relative contribution of hydroxylamine oxidation and nitrifier-denitrification to N_2O production on the basis of SP as follows.

$$\mathrm{SP_{measured}} = \mathrm{SP_{NOR}} \times X_{\mathrm{NOR}} + \mathrm{SP_{HAO}} \times (1 - X_{\mathrm{NOR}}) \quad (4)$$

In Eq. (4), $\mathrm{SP_{measured}}$, $\mathrm{SP_{NOR}}$, and $\mathrm{SP_{HAO}}$ represent SP values of observed N_2O and N_2O produced in enzymatic reaction with NOR and HAO, respectively, and X_{NOR} indicates the relative contribution of the NOR-mediated pathway. As shown in Table 4, average X_{NOR} is calculated as 82 ± 18 %, 42 ± 10 %, and 22 ± 4 % under 0, 2, and 21 % initial O_2 concentrations, respectively. These values were similar to those measured by Frame and Casciotti (2010). Using the total amount of N_2O produced in each experiment, the N_2O produced from NO_2^- reduction is estimated at 2.5 ± 1.0 nmol ($n = 6$), 3.8 ± 0.7 nmol ($n = 2$), 3.9 ± 0.7 nmol ($n = 5$) under 0, 2, and 21 % initial O_2 concentrations, respectively. This means that the rate of N_2O production via the NO_2^- reduction pathway does not depend on the amount of NO_2^- produced, which showed an increase of more than 20 times under aerobic conditions compared to anaerobic conditions. This might indicate that activities of NIR or NOR were not enhanced in this study, although we did not directly measure the regulation and activity of the enzymes. The process information provided by SP values enables us to estimate bulk ^{15}N-enrichment factors (ε, which is approximately equal to $\delta^{15}\mathrm{N_{product}} - \delta^{15}\mathrm{N_{substrate}}$ under the excess supply of substrate) for N_2O production from NH_4^+ by NOR- and HAO-mediated pathways. If N_2O is produced only by a NH_2OH oxidation pathway ($X_{\mathrm{NOR}} = 0$), $\mathrm{SP_{measured}} = \mathrm{SP_{HAO}}$ and $\delta^{15}\mathrm{N^{bulk}}$ of N_2O is estimated at -32.9 ‰ from the linear relationship between SP and $\delta^{15}\mathrm{N^{bulk}}$ (Fig. 3). However, if N_2O is produced only by a NO_2^- reduction pathway ($X_{\mathrm{NOR}} = 1$), $\mathrm{SP_{measured}} = \mathrm{SP_{NOR}}$ and $\delta^{15}\mathrm{N^{bulk}}$ of N_2O is estimated at -67.5 ‰. Combining these values with δ^{15}N of $(NH_4)_2SO_4$ used in this study ($= -0.34$ ‰), we obtain $\varepsilon_{\mathrm{HAO}} = -32.6$ ‰ and $\varepsilon_{\mathrm{NOR}} = -67.2$ ‰. The ε_{NOR} value is about 10 ‰ lower than the value estimated from pure culture incubation of *Nitrosomonas marina* C-113a (Frame and Casciotti, 2010) under several O_2 concentrations ($^{15}\varepsilon_{\mathrm{ND}} = -56.9$ ‰). The cause of the difference could be different experimental approaches (with/without enzymatic reactions) or different species studied. However, it is evident that ε is significantly different between the NH_2OH oxidation pathway and the NO_2^- reduction pathway.

Finally, we discuss the mechanisms that control SP values of N_2O produced during the enzymatic reactions. Both HAO and NOR enzymes are known to have Fe atoms as active centers, but their structures are different according to functional types. Because the catalytic site of HAO has a single Fe atom (one nuclear center) (Igarashi et al., 1997), it is likely that a single NH_2OH molecule binds to the center and is oxidized

Table 4. Estimated contribution of NO_2^- reduction catalyzed by NOR to N_2O production by *N. oceani* under different initial O_2 concentrations.

Initial O_2 (%)	N_2O (nmol)	N_2O yield (10^{-5})	X_{NOR}*	N_2O_{NOR}** (nmol)
0 %	2.9 ± 0.9	1.5 ± 0.5	0.82 ± 0.18	2.5 ± 1.0
2 %	8.9 ± 0.5	4.7 ± 0.3	0.42 ± 0.10	3.8 ± 0.7
21 %	18.1 ± 3.6	9.5 ± 1.9	0.22 ± 0.04	3.9 ± 0.7

N_2O yield represents the fraction of N atoms converted to N_2O from substrate NH_4^+ ($[N_2O\text{-}N] / [NH_4^+\text{-}N]$).

* Contribution of NO_2^- reduction to N_2O production.

** Amount of N_2O produced by NO_2^- reduction.

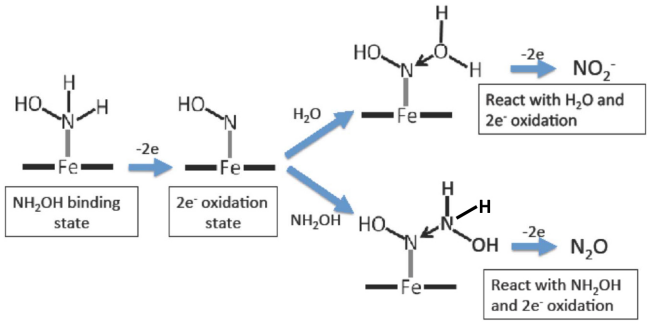

Figure 4. Two-step model of NH_2OH oxidation catalyzed by HAO.

to NO_2^-. To explain the high SP values of the N_2O generated by HAO reactions, we propose a two-step reaction mechanism as indicated in Fig. 4.

If substrate NH_2OH is supplied in excess or the electron acceptor for the reaction is lacking, however, another NH_2OH molecule will bind to the same center to form an N-N bond. In this case, the primary-binding NH_2OH molecule could be more depleted in ^{15}N than the secondary-binding NH_2OH molecule by the kinetic isotope effect. The observed positive SP might indicate that the peripheral (β) and central (α) N atoms in the product N_2O derive from the primary and secondary NH_2OH, respectively.

The catalytic center of the *N. europaea* HAO for hydroxylamine oxidation is formed by the P460 molecule and the side-chains of three amino acids (Asp291, His292, Tyr358; *N. europaea* HAO numbering) that surround the distal side of the heme (Igarashi et al. 1996). Although two of the three residues (Asp291 and His292) are completely conserved among all the HAOs, the other (Tyr358) is replaced by Asn in the enzymes of the gamma-AOBs, including the NS58. Recently, the crystal structure of the *N. europaea* HAO in the substrate-binding state was reported by Maalcke et al. (2014). They proposed that the side-chain of Tyr358 would participate in the second step of the two-step reaction shown in Fig. 4, namely the addition of water molecules to the intermediate species bound on the P460. The replacement of the Tyr to Asn in *N. oceani* NS58 HAO (Asn368) may obstruct the addition of H_2O to the intermediate in the HAO reaction. For that reason, it is probable that the N_2O-generating pathway shown in the Fig. 4 would be more likely to occur in the case of *N. oceani* NS58 HAO compared to the case of *N. europaea* HAO. In addition, this explanation is consistent with the present result that *N. oceani* NS58 generates more N_2O under the aerobic condition than the anaerobic condition (Fig 3), showing a contrast to the case of *N. europaea* (Poth and Focht., 1985).

In the case of NOR, three types are known for bacteria: cNOR that accepts electrons from cytochrome c, and qNOR and qCuNOR that accept electrons from quinols (Zumft et al. 2005). Bacterial denitrification is considered to be catalyzed by cNOR, although qNOR and qCuNOR may be responsible for detoxification of NO produced in environments (Hendriks et al. 2000). The active site of NOR enzymes has two Fe atoms (binuclear center) and have similarity among NOR types. In the case of cNOR, the binuclear center consists of non-heme iron (Fe_b) and heme b_3 (Hino et al. 2010), and it is proposed that two NO molecules respectively bind to the two centers simultaneously to form N_2O (Watmough et al. 2009). This parallel binding mechanism could bring about nearly the same isotope effect for the two N atoms in intermediate ONNO, and if the elimination of the O atom from N-O bonding does not fractionate ^{15}N within the intermediate molecule, then the SP value of N_2O would be nearly 0 ‰.

5 Conclusions

We presented direct evidence that SP values of N_2O produced by bacterial nitrification and denitrification are controlled by the enzymatic reactions of HAO and NOR during NH_2OH oxidation and NO reduction, respectively. The SP value does not depend on factors like concentration and isotope ratios of substrate and degree of reaction progress. Using the distinct SP values for HAO- and NOR-related processes, we evaluated the relative contributions from the two pathways of N_2O production by AOB in pure culture, and showed that they are sensitive to oxygen concentration and that NO_2^- reduction could occur under aerobic conditions.

Although further studies are required, this study demonstrates that isotopomer analysis constitutes a powerful tool to investigate N_2O production pathways in various environments. It will be important to expand these observations beyond nitrifying and denitrifying bacteria to examine the generality of these results. For example, isotopomeric characteristics on fungal denitrification and archaeal nitrification have been reported only recently (Santro et al., 2011; Sutka et al., 2008), and those on fungal co-denitrification are unknown. Combined analysis of several isotopomer ratios should be developed to distinguish NH_2OH oxidation and fungal denitrification, because SP values of N_2O from the two pathways have been found to be nearly the same (Sutka et al.,

2008). As for the characterization of various microbial N_2O production processes, studies focused on enzymatic reactions would be effective, as shown in this work. Although the enzymatic pathway for N_2O production by AOA is still uncertain, combined analysis of genome sequences, physiology, and isotopomer ratios would promise further understanding of microbial N_2O production mechanisms.

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