Isotopic Fractionation of Sulfur in Carbonyl Sulfide by Carbonyl Sulfide Hydrolase of *Thiobacillus thioparus* THI115

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Carbonyl sulfide (COS) is one of the major sources of stratospheric sulfate aerosols, which affect the global radiation balance and ozone depletion. COS-degrading microorganisms are ubiquitous in soil and important for the global flux of COS. We examined the sulfur isotopic fractionation during the enzymatic degradation of COS by carbonyl sulfide hydrolase (COSase) from *Thiobacillus thioparus* THI115. The isotopic fractionation constant (εvalue) was –2.2±0.2‰. Under experimental conditions performed at parts per million by volume level of COS, the εvalue for intact cells of *T. thioparus* THI115 was –3.6±0.7‰, suggesting that, based on Rees’ model, the εvalue mainly depended on COS transport into the cytoplasm. The εvalue for intact cells of *T. thioparus* THI115 was similar to those for *Mycobacterium* spp. and *Williamsia* sp., which are known to involve the conserved region of nucleotide sequences encoding the clade D of β-class carbonic anhydrase (β-CA) including COSase. On the other hand, the εvalue was distinct from those for bacteria in the genus *Cupriavidus*. These results provide an insight into biological COS degradation, which is indispensable for estimating the COS global budget based on the isotope because of the significant contribution of COS degradation by microorganisms harboring β-CA family enzymes.

**Key words:** biogeochemical processing of sulfur, sulfur isotopes in COS, GC-IRMS, sink of atmospheric COS, β-class carbonic anhydrase

Carbonyl sulfide (COS) is one of the most abundant sulfur compounds in the troposphere at nearly 500 parts per trillion by volume (pptv) (8, 53). Due to its chemical stability, some COS in the troposphere is transported to the stratosphere and is ultimately converted to sulfate by photolysis or a reaction with O or OH radical (7, 9). The resultant sulfate is one of the major sources of the stratospheric sulfate aerosols influencing the Earth’s radiation balance and ozone depletion (9, 75). On the other hand, COS has been proposed as a promising tracer to estimate gross primary production by plants (5, 70, 77). Thus, it is imperative to understand the global budget of COS in order to estimate climate change by stratospheric sulfuric acid aerosols in the future. However, estimates of COS emission and uptake values remain unclear (42).

Major sinks of atmospheric COS are vegetation and soil, and soil microorganisms, such as bacteria and fungi, are considered to play an important role in COS degradation in soil because many isolates belonging to diverse taxonomic groups exhibit strong COS-degrading abilities (48, 56). β-class carbonic anhydrase (β-CA, EC 4.2.1.1) (57), COSase (55), and carbon disulfide (CS₂) hydrolase (EC 4.4.1.27) (71, 72) exhibit high COS-degrading activities and belong to the β-CA family of enzymes, which have similar amino acid sequences and X-ray crystal structures. Among these enzymes, COSase purified from *Thiobacillus thioparus* THI115 is the only bacterial enzyme with characterized catalytic properties and an elucidated X-ray crystal structure (55). *T. thioparus* is a chemolithoautotrophic sulfur-oxidizing bacterium that widely inhabits soil and freshwater (37). *T. thioparus* THI115 was isolated from activated sludge used for the wastewater treatment of a coke-oven factory, and can grow using thiocyanate, which is an ingredient in the wastewater, as a sole energy source (34). During thiocyanate degradation, COS is produced as a reaction product of thiocyanate hydrolase (EC 3.5.5.8), and the resultant COS is then hydrolyzed to hydrogen sulfide and carbon dioxide by COSase, with COS ultimately being oxidized to sulfate (34, 35, 38, 55). Thus, COSase is an important enzyme in energy production by *T. thioparus* THI115.

An isotope analysis represents a promising tool for tracing the global dynamics of atmospheric trace gases that influence the Earth’s climate (4, 27). In order to apply an isotope analysis and interpret changes in isotopic compositions on an observational scale, the assessment of isotopic fractionation in chemical/biological processes is an essential step. In this context, studies examining isotopic fractionation in each process have involved incubation experiments with isolates or natural environmental samples. However, large variabilities have been reported in isotopic fractionation factors, even for the same reactions, due to differences in the experimental conditions used (e.g. [32, 83] for sulfur oxidation). Thus, the examination of isotopic fractionation via isolated enzymes is important for comparing and discussing the factors controlling it. To the best of our knowledge, the study of isotopic fractionation by isolated enzymes involved in biogeochemical reactions has been limited to enzymes such as RubisCO for photosynthesis (59), glycolate oxidase for photorespiration (18), nitrate reductase (33) and nitric oxide reductase (81) for denitrification, hydroxylamine oxidoreductase (81) for nitrification, nitrogenase (76) for nitrogen fixation, and glutamate
dehydrogenase (80) and glutamine synthetase (82) for ammonium assimilation. In the case of biogeochemical sulfur cycles, isotopic fractionation has only been examined using dissimilatory sulfite reductase (DsrAB), one of the important enzymes in microbial sulfate reduction (43). Although previous studies reported isotopic fractionation using isolates and/or natural samples (6, 13–16, 19, 26 [cited from 6 and 83], 28–32, 41 [cited from 83], 50, 51 [cited from 6 and 83], 54, 79, 83), an enzyme level analysis has not yet been conducted on isotopic fractionation for sulfur oxidation.

Online gas chromatograph-isotope-ratio mass spectrometry (GC-IRMS) for COS sulfur isotopic measurement was recently developed and has the ability to measure sulfur isotopic compositions at nanomole COS levels (22). By using this method, isotopic fractionation constants for COS degradation at 4,000 parts per million by volume (ppmv) COS were assessed using chemoorganotrophic COS-degrading soil bacteria (36) isolated by Kato et al. (29). Four strains of Mycobacterium spp., Williamsia sp., and two strains of Capriavidus spp. showed a preference for the degradation of CO\textsubscript{3}S over CO\textsubscript{4}S, with isotopic fractionation constant (\(\delta^34S\)) values of −3.99 to −3.56‰, −3.74‰, and −2.38 to −2.09‰, respectively. Although the number of isolates used in these experiments was limited, these findings suggest that the differences observed in \(\delta^34S\) values among the isolated bacteria are highly dependent on the genus. Although experiments at very low concentrations, such as atmospheric COS, cannot be conducted for technical reasons, elucidating the mechanisms controlling isotopic fractionation will contribute to estimations of isotopic fractionation by microbial COS degradation in natural environments. Therefore, the isotopic fractionation constants of COSase, which is a unique enzyme for COS degradation identified in the chemolithoautotrophic sulfur-oxidizing bacterium of \(T.\) thioparus THI115, as well as those of intact cells of this bacterium were assessed in the present study.

We examined the sulfur isotopic fractionation of COS by COSase in consideration of the importance of \(\beta\)-CA family enzymes to the global COS budget. Furthermore, sulfur isotopic fractionation in COS degradation by intact cells of \(T.\) thioparus THI115 was investigated in order to clarify the details of isotopic fractionation for the transport of COS into the cytoplasm and its degradation by COSase. The mechanisms underlying isotopic fractionation in bacterial COS degradation are also discussed using isotopic fractionation constants in addition to those of chemoorganotrophic bacteria reported previously (29).

Materials and Methods

Purification of COSase

COSase used in the present study was prepared as described previously (55), except that glutathione \(S\)-transferase (GST)-fused COSase bound to Glutathione Sepharose 4B (GE Healthcare, Chicago, IL, USA) was rinsed thoroughly, and the enzyme solution was filtered with a membrane filter before being applied to an ion exchange column. Briefly, the GST-fused COSase expression vector pCQ015, a COSase gene-inserted pGEX-6P-1 (GE Healthcare), was transformed into \(Escherichia\) coli Rosetta-gami B (Merck Millipore, Billerica, MA, USA). The expression of GST-fused COSase was induced by the addition of isopropyl \(\beta\)-D-1-thiogalactopyranoside to the liquid culture, and cells were lysed by lysozyme. GST-fused COSase was captured by Glutathione Sepharose 4B, precipitated at 5000g at 4°C, and rinsed thoroughly with Factor Xa Cleavage/Capture Buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM CaCl\(_2\), pH 8.0) (Merk Millipore). After the digestion by Factor Xa (Merk Millipore) and capture of Factor Xa by Xarrest Agarose (Merk Millipore), the enzyme solution was filtered using a membrane filter with a pore size of 0.45 μm (Milllex-HV Durapore PVDF membrane; Merck Millipore), then applied to a HiTrap Q HP column (1 mL, GE Healthcare). The purified enzyme solution was desalted and concentrated with 50 mM Tris-HCl, pH 8.5 using an Amicon Ultra-15 Centrifugal Filter Unit (Merk Millipore). Protein concentrations were estimated using Lowry’s method with bovine serum albumin as the standard (46).

COS-degrading experiment by COSase

A 68-ml vial (V-50; Nichiden-Rika Glass, Kobe, Japan) was sealed with a butyl rubber stopper, the headspace was replaced with nitrogen, and 3.43 mL of COS (104,000 ppmv with \(N_2\), as the balance gas; Taiyo Nippon Sanso, Tokyo, Japan) was then added via a gas-tight microsyringe to make approximately 5,000 ppmv COS. After stabilizing the inside pressure using a needle, 2 mL of the enzyme solution containing 50 μg of COSase in 50 mM Tris-HCl, pH 8.5 was added to the vial and incubated at 30°C in triplicate. A control without COSase was prepared in order to monitor the non-enzymatic degradation of COS in duplicate. Sampling of the headspace gas was started 5 min after the addition of COSase in order to avoid the disturbance of the headspace gas by the addition of the enzyme solution. Sampling of the headspace gas was performed with a gas-tight microsyringe, and 30 μL or 50 μL and 1 mL, 2 mL, or 3 mL of the gas was used for the estimation of COS concentrations and GC-IRMS measurements, respectively. The sample for GC-IRMS was transferred into a 5-mL serum bottle (V-5B; Nichiden-Rika Glass) filled with ultrahigh purity He (>99.99995%; Taiyo Nippon Sanso).

Bacterial culture

Fully grown \(T.\) thioparus THI115 (NRBC 105750) was prepared in a flask containing 10 mL of mTC10 liquid medium, mineral salts medium supplemented with thiocyanate as its sole energy source, with reciprocal shaking at 120 rpm at 30°C (34, 55). A loopful of precultured cells was inoculated on 10 mL of mTC10 agar slant medium in a glass test tube (20 cm in length, 2 cm in inner diameter). It is important to discriminate COS degradation by \(T.\) thioparus THI115 from the abiotic hydrolysis of COS with water (10, 12). Therefore, slant, not liquid medium was employed for the cultivation of the bacterium in order to reduce abiotic effects on the added COS as described below. After an incubation at 30°C for 25 d to achieve full growth, the COS-degrading experiment was performed.

COS-degrading experiment by \(T.\) thioparus THI115

After the full growth of \(T.\) thioparus THI115, the silicon sponge cap was changed to a butyl rubber stopper to seal the tube in triplicate. Based on the chemolithoautotrophic characteristics of this bacterium, the headspace of 40 mL of the tube was replaced with 80% nitrogen, 20% oxygen, and 0.03% carbon dioxide. An uninoculated control was prepared to compare COS degradation in duplicate. Tubes were incubated at 30°C and the COS-degrading reaction was initiated by injecting 104,000 ppmv COS into the headspace via a gas-tight microsyringe to make approximately 4,000 ppmv COS. Sampling of the headspace gas using a gas-tight microsyringe was started 20 min after the addition of COS in order to prevent the disturbance of the headspace gas by the addition of COS. After approximately 70% of the initially added COS was degraded, the number of cells that grew on slant medium was counted. The rate constant \(k\) (h\(^{-1}\)) of COS degradation was defined based on the degradation curve to the exponential function \(C_d(t) = C_0 e^{-kt}\), where \(C_d(t)\) is the concentration of COS at time \(t\) (h) and \(C_0\) is the initial COS concentration.

COS concentration measurements

COS concentrations were estimated by directly injecting the headspace gas into a gas chromatograph (GC-14B; Shimadzu, Japan). The COS-degrading experiment was performed.
Kyoto, Japan) as described previously (29). Briefly, the gas chromatograph was equipped with a flame photometric detector and glass column (2.0 mm in length, 3.0 mm in inner diameter) packed with Sunpak-S (Shimadzu). N₂ gas (Ichimura Sanso, Tokyo, Japan) was used as the carrier gas at a flow rate of 100 mL min⁻¹. The temperatures for the injector, column, and detector were 190°C, 60°C, and 190°C, respectively. COS concentrations were corrected by taking into account decreases caused by sampling. The relative standard deviation was within 3%.

**Stable isotope measurement**

The sulfur isotopic composition of COS was measured using online GC-IRMS with measurements of the fragment ion S from COS, as developed previously (22). Briefly, the carrier gas was He (>99.99995% purity; Taiyo Nippon Sanso) treated with a 5 A Molecular Sieve (Sigma-Aldrich, Tokyo, Japan) to remove any trace contaminants. An aliquot (an amount larger than 8 nmol) of COS in a 5-mL serum bottle was initially trapped in a stainless-steel tube chilled with liquid N₂. COS was then desorbed by bringing back to room temperature and transferred by a He flow to a capillary tube chilled with liquid N₂. Preconcentrated COS was desorbed by removing the tube from liquid N₂ and introduced into the GC equipped with a capillary column (30 m in length, 0.32 mm in inner diameter, and with a thickness of 10 μm, HP-Plot Q; Agilent Technologies, Santa Clara, CA, USA), and then IRMS (MAT 253; Thermo Fisher Scientific, Bremen, Germany) was performed to measure the fragment ions 32S, 33S, and 34S. Approximately 11 ppmv of COS (Japan Fine Products, Kawasaki, Japan) was used to confirm the accuracy of the measurements and the standard deviations for δ32S, δ33S, and Δ34S values of the triplicate measurements were within 1.0%, 0.1‰, and 0.1% during the experimental period of COSase, and 0.6‰, 0.3‰, and 0.5‰ during the experimental period of T. thioparus THI115, respectively.

**Cell number**

Living and dead cells were enumerated microscopically after live/dead staining (Live/Dead BacLight Bacterial Viability Kit for microscopy; Thermo Fisher Scientific), as described previously (29). Briefly, cells on slant medium were suspended in 0.85% NaCl, collected by centrifugation at 10,000 x g for 15 min, and then rinsed twice as described. The pellet was resuspended in 0.85% NaCl and Live/Dead reagent was added to 1 mL of the diluted cell suspension. After 15 min, the cell suspension was filtered onto a polycarbonate black filter (0.2 μm pore size and 25 mm in diameter; Advantec Toyo Kaisha, Tokyo, Japan) treated with 0.01% poly-L-lysine (P8920; Sigma-Aldrich). The cell number was enumerated under a fluorescence microscope (BZ-8000; KEYENCE, Osaka, Japan).

**Definition**

The isotopic compositions of 33S and 34S values were expressed in δ notations as

\[ \delta^S = \frac{R_{sample}}{R_{reference}} - 1 \]

where \( R_{sample} \) and \( R_{reference} \) are the isotope ratios (\(^S\)/32S, where \( x = 33 \) or 34) of residual COS at the times indicated and initial COS, respectively. In addition, \( \Delta^{34}S \) value was used to distinguish between mass-dependent fractionation (MDF) and mass-independent fractionation (MIF; or non-mass-dependent fractionation) and defined as deviations from the MIF line, as expressed in the following equation:

\[ \Delta^{34}S = \delta^{34}S - [(\delta^{34}S + 1)^{0.515} - 1] \]

The magnitude of isotopic fractionation during a single unidirectional reaction was expressed using the isotopic fractionation factor \( \alpha \),

\[ \alpha = \frac{k^x}{k^y} \]

where \( \epsilon^x (x = 33 \) or 34) and \( \epsilon^y \) are the reaction rates for the heavy and light isotopes, respectively. The isotopic fractionation constant \( \epsilon^x \) (\( x = 33 \) or 34) is defined as follows:

\[ \epsilon^x = (\alpha - 1) \]

On the other hand, \( \epsilon^x \) can be estimated from the Rayleigh equation according to Mariotti et al. (47),

\[ \delta^S - \delta^{S}_{initial} = \epsilon^{lnf} \]

where \( f \) is defined as the ratio of the residual COS concentration at the times indicated divided by the initial COS concentration. MIF can be described as a deviation from MDF in \( ^{34}S \) (\( ^{34}E \)) defined by the MDF law (1, 24), as follows:

\[ ^{34}E = \frac{33\epsilon - 0.515\epsilon^3}{32\epsilon} \]

**Statistical analysis**

The rate constant \( k \) of COS degradation was calculated using the function SLOPE from Excel for Mac 2011 (version 14.7.2, Microsoft, Redmond, WA, USA). Free software R (version 3.4.1) (R Core Team. 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/) was used to calculate and analyze statistics for the \( \epsilon^x \) and \( ^{34}E \) values for COS degradation by COSase and T. thioparus THI115. The slope between \( ^{34}S \) or \( ^{34}E \) values and lnf shows the \( \epsilon \) value, and the \( \epsilon \) value±SD was calculated based on the least square method passing through an origin using the function summary from R. The resultant values were shown as the mean±SD in triplicate. A t-test was performed for the comparison of \( \epsilon \) values between COSase and T. thioparus THI115 using the function t.test from R. \( P \) values<0.05 were considered to indicate significance.

**Results and Discussion**

**Sulfur isotopic fractionation for COS degradation by COSase**

COSase degraded more than 70% of the initially supplemented COS within 2 h under the experimental conditions employed (Fig. 1A). Degradation curves fit the exponential function and the rate constant was 0.79±0.10 h⁻¹ (\( r^2=1.00 \)), indicating that COS degradation by COSase was a first order reaction within the range of the COS concentrations used in the present study (Fig. 1A and Table 1). On the other hand, control batches without COSase showed slight variations in COS concentrations (Fig. 1A). Since no H₂S was detected, we attributed these variations to experimental errors, not chemical hydrolysis. COS degradation by COSase relative to the control batches was very fast; therefore, variations were not considered to affect the assessment of isotopic fractionation. The \( \delta^{34}S \) and \( \Delta^{34}S \) values in residual COS increased during COS degradation, and the slope between \( \delta^{34}S \) or \( \Delta^{34}S \) values and lnf showed that \( \epsilon^3 \) and \( \epsilon^4 \) values were –1.0±0.1‰ and –2.2±0.2‰, respectively (Fig. 1B and C, and Table 1).

The purified enzymes studied to date in sulfur isotopic fractionation have only been DsrAB and s-triazine hydrolase (TrzN) (43, 67), and reported \( ^{34}E \) values were –15.3‰ and –16‰ for sulfite reduction by DsrAB from Desulfovibrio vulgaris and Archaeoglobus fulgidus, respectively. The \( ^{34}E \) value for TrzN from Arthrobacter aurescens was –14.7‰ for the substitution of the thiomethyl group in the herbicide ametryn to a hydroxyl group. In contrast to these enzymes, COSase showed smaller isotopic fractionation. The active
Fig. 1. Time courses of COS and sulfur isotopic compositions during COS degradation by COSase (A, B and C) and T. thioparus THI115 (D, E and F). ln/$f$ represents the natural logarithm of the ratio of the residual COS concentration at the times indicated divided by the initial COS concentration. (A and D) ●, ○, and □ represent batches 1, 2, and 3 of COSase or T. thioparus THI115, respectively. × and + represent batches 1 and 2 of buffer without COSase or the uninoculated control, respectively. (B, C, E and F) ●, ○, and □ represent batches 1, 2, and 3 of T. thioparus THI115 or COSase, respectively. The COS concentration at 0 min cannot be measured because of the disturbance of the headspace gas by the addition of COS. Therefore, the concentration of T. thioparus THI115 at 0 min was regarded as those of batches 1 and 2 of the uninoculated control measured at 20 min and 60 min, respectively, corresponding to the date of the measurement.

Table 1. Sulfur isotopic fractionations in COS degradation by COSase and T. thioparus THI115.

| Batch | Isotope sampling times | Rate constant (h$^{-1}$) | Living cell number ($\times 10^9$ cell) | Cell specific activity ($\times 10^{-10}$ h$^{-1}$ cell$^{-1}$) | $^{33}$S | $^{34}$S | $^{33}E$ (%) |
|-------|------------------------|--------------------------|----------------------------------------|-------------------------------------------------|--------|--------|----------|
|       |                        |                          |                                        |                                                  | $r^2$   | $P$ value | $r^2$ | $P$ value |
| COSase | 1                      | 5                        | 0.90                                   | —                                               | —      | 0.99    | $2.8\times10^{-5}$ | 0.1 |
|        | 2                      | 5                        | 0.72                                   | —                                               | —      | 1.00    | $7.6\times10^{-5}$ | 0.2 |
|        | 3                      | 5                        | 0.75                                   | —                                               | —      | 0.99    | $4.1\times10^{-5}$ | 0.1 |
|        | Average$^b$             | —                        | 0.79±0.10                              | —                                               | —      | —      | —        | — |
| T. thioparus | 1                  | 6                        | 0.36                                   | 4.7                                             | 0.76   | —      | $9.1\times10^{-6}$ | 0.1 |
| THI115 |                        |                          |                                        |                                                  | —      | 0.99    | $2.9\times10^{-6}$ | 0.1 |
|        | 2                      | 5                        | 0.30                                   | 2.0                                             | 1.51   | —      | $2.0\times10^{-6}$ | —2 |
|        | 3                      | 6                        | 0.33                                   | ND                                             | ND     | —      | $1.3\times10^{-5}$ | —3 |
|        | Average$^b$             | —                        | 0.33±0.03                              | 3.3±1.9                                         | 1.1±0.5 | —      | $3.9\times10^{-7}$ | 0.3 |

ND, not determined. $^a$ Indicated ±SD. $^b$ Indicated mean±SD calculated based on the value of each batch. $^c$ $P$ values for $e$ values between COSase and T. thioparus THI115 were 0.08 for the $^{33}$e value and 0.03 for the $^{34}$e value.
Sulfur Isotopic Fractionation by COS Hydrolase

site of TrzN had a similar X-ray crystal structure to that of α-CA; however, their amino acid sequences and overall folding structures were different (69). The conformations of the active site between β-CA including COSase and α-CA are similar to each other; however, the COS-degrading activity of α-CA is lower than that of β-CA (20, 39, 57). Although the substrates for COSase and TrzN differ, a common reaction mechanism has been proposed for these enzyme proteins, as follows: a nucleophilic attack to the carbon atom by the hydroxide ion coordinated with the zinc ion at the active site, followed by cleavage of the C-S bond in the substrate (55, 67). Schürner et al. (67) proposed that C-S bond cleavage, which was rate-limiting for the energetic barrier, was the reason for the large sulfur isotope effect by TrzN. On the other hand, a computational model in the reaction of COS with [(H₂N)₂ZnOH]⁺, a biomimetic complex used to mimic the active site of α-CA, showed that the rate-limiting step was the nucleophilic attack (66). Therefore, we hypothesize that the factor controlling isotopic fractionation by COSase may be the rate-limiting reaction by the nucleophilic attack to the carbon atom of COS. In other words, since cleavage of the C-S bond in COS was not the rate-limiting step in the hydrolysis of COS, cleavage mostly proceeded and did not show isotopic fractionation for the cleavage reaction itself.

COS degradation by T. thioparus THI115

*T. thioparus* THI115 degraded approximately 70% of initial COS in 4 h (Fig. 1D). Cell numbers on slant medium in culturing tubes supplemented with COS were in the range of 4.7×10⁵ to 2.0×10³ cells tube⁻¹ for living cells and 4.8×10⁶ to 1.3×10⁸ cells tube⁻¹ for dead cells. Cell numbers in the COS free slant tube were in the range of 3.1×10⁸ to 3.2×10⁹ cells tube⁻¹ for living cells and 8.1×10⁸ to 1.8×10⁹ cells tube⁻¹ for dead cells, suggesting that cells were not negatively affected by the COS treatment. Degradation curves were fit to the exponential function and the rate constants were 0.33±0.03 h⁻¹ (r²=0.94), showing that COS degradation is a first order reaction (Fig. 1D and Table 1). The cell-specific activities (rate constant divided by cell number) of *T. thioparus* THI115 were in the range of 0.76 to 1.51×10⁻⁴ h⁻¹ cell⁻¹ (Table 1). Among bacteria examined to date, *T. thioparus* THI115 has exhibited the highest COS-hydrolyzing activity; the specific activities of bacterial isolates already examined have been in the range of 0.07 to 1.12×10⁻¹ h⁻¹ cell⁻¹ (29). On the other hand, un inoculated control batches showed variations in COS concentrations, as did control batches for COS degradation by COSase. However, H₂S was not observed in this case, and the reason for the variations observed beyond the measurement error currently remains unclear. The isotopic fractionation of an abiotic decrease in COS was not assessed because COS degradation by *T. thioparus* THI115 was markedly faster than that by the control batches.

*T. thioparus* THI115 is a sulfur-oxidizing bacterium that degrades COS and H₂S as the chemolithotrophic energy source. This bacterium also tolerates high concentrations of COS because the addition of 1,500 ppmv and 25,000 ppmv of COS did not negatively affect the degradation of thiocyanate or COS, respectively (38). The high activity observed by *T. thioparus* THI115 is appropriate because the COS-degrading reaction is associated with the energy production pathway for this bacterium. On the other hand, the physiological functions of the COS-degrading activity of various chemoorganotrophic bacteria (36, 55) and fungi (44, 48) have not yet been elucidated in detail. Approximately half of these bacteria contain a gene of clade D of β-CA, one of four clades into which β-CA is phylogenetically classified (73, 74). COS degradation may only accompany the reversible hydration of CO₂, the natural substrate of β-CA, to HCO₃⁻ and H⁺ by β-CA because COS is a structural analog of CO₂.

Sulfur isotopic fractionation for *T. thioparus* THI115

δ³⁴S and δ³³S values for residual COS increased during COS degradation by *T. thioparus* THI115, as was also the case for COSase (Fig. 1E and F). The δ³⁴S and δ³³S values estimated from the slope were −1.8±0.6‰ and −3.6±0.7‰, respectively (Fig. 1E and F, and Table 1), while δ³³S values were 0.1±0.1‰ for COSase and 0.1±0.2‰ for *T. thioparus* THI115 (Table 1). We herein demonstrated for the first time that δ³⁴S values for *T. thioparus* THI115 and COSase were different (P value<0.05). Most errors in isotope ratio measurements were caused by MDF, and variations in δ³³S values were less than those for δ³⁴S and δ³⁴S values (Table 1). Since the interpretation of δ³³S values will be discussed in the next section, we only focused on the δ³⁴S values for *T. thioparus* THI115 and COSase in this section.

In order to elucidate the mechanisms and factors controlling sulfur isotopic fractionation for COS degradation, we considered Rees’ model (62). The cell membrane is one of the important factors for isotopic fractionation by diffusion and/or active transport (21, 58). Based on Rees’ model, isotopic fractionation in COS degradation by intact *T. thioparus* THI115 reflects effects on COS transport into the cytoplasm, the hydrolysis of COS by COSase, and the ratio of efflux to influx [kₜₒᵤₜ/(kₒᵤₜ+ kₑᵤₜ)] or [kₑᵤₜ/kₒᵤₜ] (Fig. 2). This relationship can be expressed using Rees’ model (62), as follows:

\[ ε_{ₙₑₜ} = ε_{ₜₒᵤₜ} + (ε_{ₑᵤₜ} - ε_{ₜₒᵤₜ})kₒᵤₜ/(kₒᵤₜ + kₑᵤₜ) \]

where \( ε_{ₑᵤₜ} \) and \( ε_{ₜₒᵤₜ} \) are isotopic fractionation constants for the overall net, transport into the cytoplasm, and the enzyme reaction, respectively. \( kₒᵤₜ \) and \( kₑᵤₜ \) are rate constants for transport into and out of the cell and for COS degradation by an enzyme, respectively. Transport is regarded as diffusion into and out of the cytoplasm through the cell wall and cell

\[ 34 \]
membrane because COS is a gaseous sulfur compound and the cytoplasm is in the liquid phase. Diffusivity in the liquid phase of one gas in another is noted as \( D_f \), and is calculated as follows (52):

\[
D_f = \left( \frac{m_1 m_2}{m_1 + m_2} \right)^{\frac{1}{2}}.
\]

In this equation, \( m_1 \) and \( m_2 \) represent the masses of a gas and liquid, respectively. Thus, \( D(\text{CO}^{32}\text{S}) \) and \( D(\text{CO}^{34}\text{S}) \) can be calculated based on the masses of \( \text{CO}^{32}\text{S}, \text{CO}^{34}\text{S}, \) and \( \text{H}_2\text{O}. \) The \( \Delta^{34}\text{c}_{\text{eff}} \) value for COS diffusion in liquid water is calculated as \( D(\text{CO}^{34}\text{S})/D(\text{CO}^{32}\text{S}) = 1 \), and the value is \(-3.7\%o\). Thus, the model shows the upper limit of \( \Delta^{34}\text{c}_{\text{net}} = -3.7\%o \) when the ratio of efflux to influx is equivalent to 0 (i.e. \( k_{\text{out}} < k_{\text{in}} \)), while the lower limit of \( \Delta^{34}\text{c}_{\text{net}} = -2.2\%o \), \( \Delta^{34}\text{c} \) value of COSase, when the ratio of efflux to influx is equal to 1 (i.e. \( k_{\text{out}} > k_{\text{in}} \)). Thus, the \( \Delta^{34}\text{c}_{\text{net}} \) value of \(-3.6\% < \Delta^{34}\text{c}_{\text{net}} < 0\%o \) obtained in the present experiment using \( T. thioparus \) THI115 suggests that the ratio of efflux to influx of this experiment was 0.07, \( k_{\text{out}} \) was 0.35 h\(^{-1}\), and \( k_{\text{in}} \) was 0.02 h\(^{-1}\). In other words, the main factor controlling the \( \Delta^{34}\text{c} \) value of bacterial COS degradation was not the enzyme, but diffusion/transport into the cytoplasm. We are the first to show enzymatic reaction by COSase, and the ratio of efflux to influx.

Isotopic fractionation for COS degradation was not the enzyme, but diffusion/transport into the cytoplasm. We are the first to show enzymatic reaction by COSase, and the ratio of efflux to influx.

Based on the emission rate of 13 pmol m\(^{-2}\) s\(^{-1}\), indicating that the lower limit of the \( \Delta^{34}\text{c} \) value for bacteria is closely related to the presence of relevant enzymes.

Fig. 3 shows a three-isotope plot represented from the results of the present study, and slopes ranged between 0.43 and 0.56. Since most errors in isotope ratio measurements were caused by MDF, variations in \( 3\text{E} \) values were less than those for \( \Delta^{34}\text{c} \) and \( \Delta^{34}\text{c} \) values (Table 1). \( 3\text{E} \) values were 0.1±0.1\%o for COSase and 0.1±0.2\%o for \( T. thioparus \) THI115 (Table 1), showing no significant difference.

Sulfur isotope anomalies originating from the MIF process have been observed in aerosols from the troposphere and in snow pits and ice cores in which aerosols from the stratosphere have been deposited (17, 63, 64). The reactions by COSase and \( T. thioparus \) THI115 as well as chemoorganovo-
Sulfur Isotopic Fractionation by COS Hydrolase

Ministry of Education, Culture, Sports, Science and Technology, Japan. no. 23310051, no. 16H05884, no. 17H06105, no. 17J08979) from the Ported in part by Grants-in-Aid for Scientific Research (no. 18310020, no. 23310051, no. 16H05884, no. 17H06105, no. 17J08979) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Fig. 3. A three-isotope plot between δ33S and δ34S values. The broken line indicates the mass-dependent fractionation line with slope (0.515). +, ×, and □ represent batches 1, 2, and 3 of COSase, respectively. ●, ○, and □ represent batches 1, 2, and 3 of *T. thioparus* THI115, respectively.

trophic bacteria reported previously did not show MIF, which confirmed that these reactions do not contribute to sulfur isotope anomalies in the atmosphere (17, 63, 64).

Conclusion

Isotopic fractionation for COSase and *T. thioparus* THI115 in the present study indicated biological effects on δ34S value of tropospheric COS and provides insights into biological COS degradation, which is important for estimating the COS global budget. In the present study, we used a purified enzyme of COSase and intact cells of *T. thioparus* THI115 in order to obtain a clearer understanding of isotopic fractionation in COS degradation by bacteria. Based on Rees’ model, we assumed that isotopic fractionation by *T. thioparus* THI115 was mainly influenced by the transport of COS into the cytoplasm during the degradation of COS, even at very low concentrations such as atmospheric COS.

Further studies with a focus on isotopic fractionation by plants, another major sink of atmospheric COS, are indispensable for distinguishing COS fluxes between soil and plants. Moreover, measurements at low COS concentrations, such as atmospheric concentrations, are needed for soil, the second largest sink of COS, and COS-degrading organisms in order to clarify the COS dynamics operating between the troposphere and soil or organisms (42). Therefore, a rapid and simple analytical method with the ability to measure isotopic fractionation at atmospheric COS levels needs to be developed.

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