

Inhibition of Ammonia Oxidation in *Nitrosomonas europaea* by Sulfur Compounds: Thioethers Are Oxidized to Sulfoxides by Ammonia Monooxygenase

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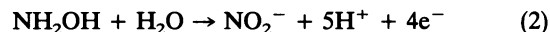
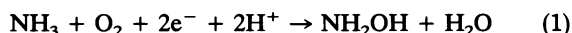
Organic sulfur compounds are well-known nitrification inhibitors. The inhibitory effects of dimethylsulfide, dimethyldisulfide, and ethanethiol on ammonia oxidation by *Nitrosomonas europaea* were examined. Both dimethylsulfide and dimethyldisulfide were weak inhibitors of ammonia oxidation and exhibited inhibitory characteristics typical of substrates for ammonia monooxygenase (AMO). Depletion of dimethylsulfide required O₂ and was prevented with either acetylene or allylthiourea, two inhibitors of AMO. The inhibition of ammonia oxidation by dimethylsulfide was examined in detail. Cell suspensions incubated in the presence of ammonia oxidized dimethylsulfide to dimethyl sulfoxide. Depletion of six other thioethers was also prevented by treating cell suspensions with either allylthiourea or acetylene. The oxidative products of three thioethers were identified as the corresponding sulfoxides. The amount of sulfoxide formed accounted for a majority of the amount of sulfide depleted. By using gas chromatography coupled with mass spectrometry, allylmethylsulfide was shown to be oxidized to allylmethylsulfoxide by *N. europaea* with the incorporation of a single atom of ¹⁸O derived from ¹⁸O₂ into the sulfide. This result supported our conclusion that a monooxygenase was involved in the oxidation of allylmethylsulfide. The thioethers are concluded to be a new class of substrates for AMO. This is the first report of the oxidation of the sulfur atom by AMO in whole cells of *N. europaea*. The ability of *N. europaea* to oxidize dimethylsulfide is not unique among the ammonia-oxidizing bacteria. *Nitrosococcus oceanus*, a marine nitrifier, was also demonstrated to oxidize dimethylsulfide to dimethyl sulfoxide.

Nitrification is the bacterially mediated process in which ammonia is oxidized sequentially to nitrite and then to nitrate. The reactions are catalyzed by specialized ammonia-oxidizing bacteria, such as *Nitrosomonas europaea*, and nitrite-oxidizing bacteria, such as *Nitrobacter winogradskyi*. Nitrifying bacteria are ubiquitous components of the soil microbial population, and their activities are stimulated in agricultural soils following the application of ammonia- or urea-based fertilizers. Nitrification can lead to loss of nitrogen fertilizers by leaching of nitrate to groundwater and surface water. Additionally, microbial denitrification of the nitrate can result in the production of N-oxides. Thus, environmental and economic considerations have maintained the long-term interest in nitrification inhibitors.

Nitrification in the soil is inhibited by several sulfur compounds. For example, the sulfur-containing amino acids methionine and cysteine inhibit nitrification (5, 21, 23). However, because methionine did not inhibit ammonia oxidation by a pure culture of *N. europaea*, the inhibitory effects of methionine were suggested to result from the microbial breakdown product(s) of methionine (26). Likewise, the inhibitory effects of cysteine on nitrification were also proposed to be due to the degradation product(s) of cysteine (2, 21). Some of the known sulfur-containing degradation products which were suggested to be inhibitors of nitrification include carbon disulfide, dimethylsulfide, dimethyldisulfide, and alkyl thiols. These compounds have been confirmed to be inhibitors of nitrification (2, 4). While the inhibition of ammonia oxidation in pure cultures of *N. europaea* by carbon disulfide was characterized (15), the

inhibition of nitrification by dimethylsulfide, dimethyldisulfide, and alkyl thiols has not been characterized in pure cultures. The purpose of this study was to examine the mechanism by which dimethylsulfide and the class of sulfur compounds known as the thioethers inhibited ammonia oxidation in pure cultures of *N. europaea*.

Ammonia oxidation in *N. europaea* and other ammonia-oxidizing bacteria is initiated by the enzyme ammonia monooxygenase (AMO) (equation 1):



The hydroxylamine generated by AMO is then oxidized to nitrite by the enzyme hydroxylamine oxidoreductase (HAO) (equation 2). Electrons derived from the oxidation of hydroxylamine provide a source of reductant for AMO during steady-state ammonia oxidation (38). The oxidation of ammonia by these bacteria therefore is susceptible to inhibition through either direct effects on AMO or indirect effects on HAO or other components which are involved in supplying electrons to AMO. Inhibitors of nitrification that act on AMO typically function by one of the following mechanisms. (i) Reversible inhibitors, such as allylthiourea (ATU) and carbon disulfide, prevent ammonia oxidation by interfering with catalysis by AMO (10, 15). (ii) Alternate substrates for AMO, such as methane, ethylene, and halogenated hydrocarbons, inhibit ammonia oxidation by competing for the flux of activated O₂ (16, 18, 19, 32). (iii) Mechanism-based inactivators of AMO, such as acetylene, irreversibly inactivate AMO (20). (iv) Exposure of cells to visible light also leads to inactivation of AMO (34). Given this diversity of inhibitory mechanisms for AMO, it is not surprising that

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AMO, rather than HAO, is most commonly identified as the target enzyme for inhibitors of nitrification (3).

In this article, we characterize the mechanisms by which several sulfur-containing compounds inhibit ammonia oxidation by *N. europaea*. The target enzyme and mechanism of inhibition were identified for the class of organic sulfur compounds known as the thioethers. Several thioethers are shown to be substrates for AMO. The products of thioether oxidations are demonstrated to be the corresponding sulfoxides. This is the first report of the oxidation of the sulfur atom by AMO.

MATERIALS AND METHODS

Materials. Carbon disulfide and all the organic sulfides and sulfoxides, except for tetrahydrothiophene sulfoxide, were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. Tetrahydrothiophene sulfoxide was purchased from American Tokyo Kasei, Portland, Oreg. All of these reagents were more than 97% pure by manufacturer analysis. C_2H_2 was generated in a gas-generating bottle from calcium carbide (~80%; Aldrich Chemical Co., Inc.) as previously described (13). $^{18}O_2$ (97.7%) was supplied by MSD Isotopes, Rahway, N.J.

Growth and preparation of the cells. *N. europaea* (ATCC 19178) (1.5 liters) was grown in 2-liter shake flasks as described previously (14). Cell suspensions were harvested by centrifugation (20,000 $\times g$, 15 min) after 3 days of growth. Cells were washed and resuspended (1.5 ml) with assay buffer (50 mM NaH_2PO_4 and 2 mM $MgCl_2$, pH 7.8). Cell suspensions were stored on ice and used within 24 h of harvesting. *Nitrosococcus oceanus* was grown in batch culture (1 liter) for 14 days in medium consisting of seawater buffered (pH 8.0) with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) as described by Ward (37). Cells of *N. oceanus* were harvested by centrifugation (20,000 $\times g$, 25 min) and washed and resuspended in seawater (1 ml). Cultures of *N. europaea* and *N. oceanus* were periodically checked for contamination by streaking on nutrient agar plates.

Analytical procedures. Sulfur compounds were analyzed with a Shimadzu gas chromatograph (model GC-8A) equipped with a flame ionization detector. The compounds were separated by using a Teflon-lined stainless steel column (0.3 cm by 34 cm) containing Porapak Q (80 to 100 mesh) (Waters Associates, Inc., Framingham, Mass.) or containing 17% Carbowax 1500 on Chromosorb G (45 to 60 mesh) operated between 45 and 170°C. Resolution of dimethyl sulfoxide from dimethylsulfone was achieved with a longer (1-m) Carbowax column. The presence of formaldehyde was determined by using an enzymatic assay as described previously (32). The protein content of the cell suspensions was determined by using the biuret assay (8) after solubilizing the cells in 3 M NaOH (45 min, 75°C). Bovine serum albumin was used as the protein standard.

Inhibition of nitrite production by volatile sulfur compounds, reversibility of the inhibition, and depletion of the compounds by cells of *N. europaea*. Stock solutions of the sulfur compounds were prepared daily by the addition of the compounds to glass serum vials filled with assay buffer and sealed with Teflon-lined silicone septa (Alltech Associates, Inc., Deerfield, Ill.). Solutions of carbon disulfide were prepared in dimethyl sulfoxide. Incubations were conducted in serum vials (9.4 ml) containing assay buffer (0.95 ml) and either ammonium sulfate (5 mM) or hydroxylamine hydrochloride (2 mM) and sealed with Teflon-lined silicone septa.

After the addition of the sulfur compound to the assay vial and equilibration for approximately 2 min at 30°C in a shaking water bath, the reactions were initiated by the addition of an aliquot of the cell suspension (0.05 ml, 0.7 mg of protein) to the assay vial. The assay vials were allowed to incubate (5 or 10 min) in the shaking water bath. Liquid samples (5 or 10 μ l) of the reaction mixtures were removed for determination of the nitrite content by colorimetric analysis as described previously (9). The rate of nitrite production from either ammonium sulfate or hydroxylamine hydrochloride in the absence of any added sulfur compound remained constant during the time course of the assays.

To examine the reversibility of any inhibition by these sulfur compounds, cells were recovered from the reaction mixture by sedimentation (14,000 $\times g$, 5 min). After three cycles of sedimenting and washing with assay buffer (3 \times 1.5 ml), the cells were resuspended in assay buffer (0.5 ml). A portion (100 μ l) of this washed cell suspension (0.14 mg of protein) was then added to assay buffer (1.8 ml) in the chamber of an O_2 electrode. The ammonia-dependent O_2 uptake was measured in the presence of ammonium sulfate (2.7 mM). ATU (100 μ M) was then added to the electrode chamber to inhibit further ammonia-dependent O_2 uptake, and then hydrazine (750 μ M) was added to the electrode chamber and the O_2 uptake rate was recorded. The ammonia- and hydrazine-dependent O_2 uptake rates of cells exposed to the sulfur compounds were compared with the rates obtained with cells which were not exposed to the sulfur compounds but were otherwise treated in the same manner.

Organic sulfide depletion assays. Stock solutions of the sulfides were made fresh daily as described above except that buffered seawater (25 mM HEPES, pH 7.8) was used for assays involving *N. oceanus*. Incubations were conducted as described above in assay buffer (0.9 ml), with the following changes: (i) the concentration of ammonium sulfate was 2.5 mM, (ii) butyl rubber septa were used for the assay vials containing dimethylsulfide, and (iii) buffered seawater replaced the assay buffer for incubations with *N. oceanus*. After the addition of the sulfide to the incubation vial and equilibration for approximately 2 min at 30°C in a shaking water bath, the reactions were initiated by the addition of the cell suspension (0.1 ml, 0.5 to 2 mg of protein) to the assay vials. To determine the amount of sulfide depleted from the reaction vials, a sample of the gas phase (25 to 50 μ l) was removed prior to the addition of cells and at designated times during the assay. At 30 min, a liquid-phase aliquot (50 μ l) was withdrawn from the reaction vials and frozen (-20°C) for product analysis at a later time. Control vials for depletion analysis contained (i) cell suspensions treated with either C_2H_2 (5 to 20 μ mol) or ATU (100 μ M) to inhibit AMO or (ii) boiled cells to assess abiotic loss.

Incorporation of $^{18}O_2$ into allylmethylsulfide by *N. europaea*. Cell suspensions, assay buffer, and the stoppered reaction vials (9.4 ml) containing assay buffer (1.2 ml) and ammonium sulfate (2.5 mM) were repeatedly evacuated and flushed with N_2 to remove O_2 . An O_2 -free stock solution of allylmethylsulfide was made by adding pure allylmethylsulfide (5 μ l) to a vial filled with anaerobic assay buffer. An aliquot of this stock solution (1.5 μ mol, 0.4 ml) was added to each of the N_2 -filled stoppered reaction vials. Two milliliters of either $^{16}O_2$ or $^{18}O_2$ (97.7%) was then added to the reaction vials as an overpressure. Reactions were initiated by the addition of the cell suspension (0.2 ml, 4 mg of protein) to the vials. The control vial contained 2 ml of $^{18}O_2$ and cell suspensions in which AMO was inactivated by treatment with C_2H_2 . The partial pressures of mass 36 ($^{18}O_2$) and mass

TABLE 1. Inhibition of ammonia oxidation in *N. europaea* by volatile sulfur compounds

Sulfur compound ^a	Amt added (μmol)	% Nitrite produced (relative to uninhibited cells) from ^b :		% Reversibility relative to uninhibited cells for ^c :		% Depletion relative to amt added ^d
		10 mM NH ₄ ⁺	2 mM NH ₂ OH	AMO	HAO	
CS ₂	0.1	23	95	16	105	ND ^e
DMS	0.5	86	101	103	93	30
DMDS	0.5	76	107	99	102	69
C ₂ H ₅ SH	0.5	0	64	19	102	63

^a Abbreviations: CS₂, carbon disulfide; DMS, dimethylsulfide; DMDS, dimethyldisulfide; C₂H₅SH, ethanethiol.

^b The percentages are expressed relative to the amount of nitrite produced by uninhibited cell suspensions incubated in the presence of either ammonium sulfate or hydroxylamine hydrochloride during a fixed time assay. The relative errors among the percentages ranged from ±1% to ±10% for the triplicate samples.

^c The cell suspensions were sedimented and resuspended three times with buffer to remove the compound. The activities of AMO and HAO were measured as the rates of ammonia- and hydrazine-dependent O₂ uptake. The activities are expressed as a percentage relative to a rate of 32.9 μmol of O₂ consumed h⁻¹ mg of protein⁻¹ for the activity of AMO or 8.2 μmol of O₂ consumed h⁻¹ mg of protein⁻¹ for the activity of HAO of the uninhibited cell suspensions.

^d Net percentage of the sulfur compound depleted by the cell suspensions during the incubation with respect to the amount of compound initially added. Background loss of the sulfide was accounted for by incubation of the compounds with boiled cell suspensions. Errors ranged from ±1% to ±4% for depletion assays performed in triplicate.

^e ND, not determined.

32 (¹⁶O₂) in the headspace of the vials were determined before the addition of the cell suspension and at the end of the incubation by using a Dycor quadrupole residual gas analyzer (Ametek, Pittsburgh, Pa.) fitted with a fused silica capillary tubing (50-μm inside diameter) as the inlet. After a 75-min incubation in a shaking water bath, the liquid phases of the reaction vials were transferred to test tubes containing sufficient solid NaCl (~0.1 g) to saturate the aqueous phase. Two extractions with ~1 ml of CH₂Cl₂ were made to remove the organic oxidation product(s). The extracted samples were then dried with excess Na₂SO₄ in preparation for analysis by gas chromatography-mass spectrometry (GC-MS). The CH₂Cl₂ extractions were separated on a fused silica capillary column (30 m, 0.25-mm inside diameter) coated with SE-54 (film thickness, 0.25 μm). The initial column temperature was 40°C for 5 min; the temperature was then increased to 275°C at a rate of 10°C/min. The injector and detector temperatures were 200°C. Mass spectral analysis of the peaks separated by using this column were obtained with a Finnigan-4023 GC-MS functioning in the electron impact mode at 70 eV. Allylmethylsulfoxide was prepared by oxidation of the allylmethylsulfide with hydrogen peroxide (30). Pure allylmethylsulfide (~0.3 mmol) at room temperature was oxidized by the dropwise addition of cold 30% H₂O₂ (~2 mmol) during vortexing (~3 min). This mixture was then diluted ~10⁴-fold with water. A portion of this mixture was then extracted into CH₂Cl₂ as described for the cellular extractions and analyzed by GC-MS.

RESULTS

Inhibition of ammonia oxidation by *N. europaea* by volatile sulfur compounds. The inhibition of nitrite production from either ammonium sulfate or hydroxylamine hydrochloride was tested for each of four sulfur compounds (Table 1). All four compounds were inhibitors of ammonia oxidation by *N. europaea*; dimethylsulfide and dimethyldisulfide were only weak inhibitors, while carbon disulfide and ethanethiol were strong inhibitors of the oxidation of ammonia to nitrite. Dimethyl sulfoxide (73 μmol), which was used to deliver carbon disulfide, did not inhibit the oxidation of ammonia or hydroxylamine to nitrite by *N. europaea* (data not presented). Carbon disulfide inhibited only the oxidation of ammonia to nitrite and not the oxidation of hydroxylamine. In contrast to the other sulfur compounds, only ethanethiol inhibited hydroxylamine oxidation to nitrite, although its

effect on ammonia oxidation was greater than its effect on hydroxylamine oxidation.

The mechanism by which each compound inhibited ammonia oxidation was investigated further by determining (i) the extent of consumption of the sulfur compound during incubation with cells and (ii) the reversibility of the inhibition of the activities of AMO and HAO. During a 10-min incubation, cell suspensions of *N. europaea* consumed 30% (dimethylsulfide), 69% (dimethyldisulfide), and 63% (ethanethiol) of the 0.5 μmol of each sulfur compound initially present in the incubation vial. Because the consumption of dimethylsulfide and dimethyldisulfide was prevented by treating cell suspensions with acetylene (C₂H₂), a specific inactivator of AMO, the depletion of these two sulfur compounds was likely due entirely to oxidation by AMO. In contrast, depletion of ethanethiol exhibited a 20% greater loss when AMO was inactivated compared with depletion by boiled cell suspensions. These results suggested that another mechanism for depletion of ethanethiol, other than and in addition to AMO, may be present in the cells. Carbon disulfide is not believed to act as a substrate for AMO (15), and therefore depletion of carbon disulfide by *N. europaea* was not determined.

The reversibility of the inhibition was determined by removing the inhibitor from the cell suspensions and measuring the residual activities of AMO and HAO as ammonia- and hydrazine-dependent O₂ uptake, respectively. The activities of AMO and HAO following incubation with either dimethylsulfide or dimethyldisulfide were essentially identical to the corresponding activities of the uninhibited cell suspensions. In contrast, after removal of carbon disulfide or ethanethiol, the ammonia-dependent O₂ uptake rates of the cell suspensions were, respectively, 16 and 19% of the rate of cell suspensions incubated in the absence of any inhibitor. However, the activity of HAO was unaffected after removal of carbon disulfide or ethanethiol, suggesting an element of specificity to the inhibition of ammonia oxidation by these compounds. Our activities for AMO and HAO following treatment with carbon disulfide corresponded well with previously published results (15).

Our characterization of four volatile sulfur compounds with regard to ammonia oxidation revealed the following. (i) Carbon disulfide was a specific and potent inhibitor of AMO, as was previously demonstrated (15). (ii) Ethanethiol was a potent inhibitor of AMO, and depletion of ethanethiol suggested that it may be a substrate of AMO. However, the

inhibitory properties of ethanethiol (partial reversibility of AMO activity, potency of inhibition on nitrite production from hydroxylamine hydrochloride, and depletion of ethanethiol in the absence of AMO activity) suggested a more complex interaction of this compound with *N. europaea* than can be explained simply by oxidation of the ethanethiol by AMO. These complex inhibitory effects of ethanethiol on *N. europaea* were not further investigated. However, the general properties of toxicity of alkyl thiols for biological systems are noted (40). (iii) Both dimethylsulfide and dimethyldisulfide were depleted by cells of *N. europaea*, which suggested that these sulfur compounds may be substrates of AMO. AMO is known to oxidize several hydrocarbons and might be expected to oxidize the methyl groups in these compounds. Although the sulfur atom has not been demonstrated to be a site of oxidation by AMO, this is not an uncommon site for oxidation by other monooxygenases (27, 28, 41).

Requirements for dimethylsulfide depletion and the formation of dimethyl sulfoxide by *N. europaea*. Two approaches were used to establish a role for AMO in dimethylsulfide consumption. First, the requirements for depletion of dimethylsulfide and those for AMO catalysis were compared. As shown in equation 1, both a source of O_2 and a reductant are required for AMO activity. Cell suspensions incubated anaerobically in the presence of ammonia did not consume dimethylsulfide. Upon reintroduction of O_2 , the cell suspensions resumed consumption of dimethylsulfide at about the same rate as did cell suspensions in the presence of O_2 (data not shown). These results indicated that O_2 was required for dimethylsulfide depletion. Cell suspensions more rapidly consumed dimethylsulfide in the presence of ammonia than in the absence of ammonia (Fig. 1A). Presumably, more reductant was available for rapid oxidation of dimethylsulfide by AMO when ammonia was present because the reductant for AMO-catalyzed reactions *in vivo* is provided by ammonia-dependent hydroxylamine oxidation. Hydrazine, an alternate substrate for HAO, can also provide a reductant for AMO-catalyzed reactions (18, 19). The addition of hydrazine (1 mM) stimulated the rate of dimethylsulfide depletion (data not shown). Cell suspensions incubated with dimethylsulfide in the absence of a reductant source slowly consumed dimethylsulfide. A supply of endogenous reductant for AMO catalysis has been proposed for *N. europaea* (38). In a second approach, the effect on depletion of dimethylsulfide by two specific inhibitors of AMO, ATU and C_2H_2 , was examined (Fig. 1A). Both inhibitors of AMO prevented ammonia-dependent consumption of dimethylsulfide (results for ATU not presented). Depletion of dimethylsulfide was also inhibited by acetylene when hydrazine was supplied as the only reductant source (data not shown). The results from both approaches were consistent with a role for AMO in dimethylsulfide consumption.

In previous studies of alternate substrate oxidations by AMO, the products of the oxidations were not metabolized by the cell suspensions and accumulated in the reaction media (18, 19, 32). On the basis of the results with microsomal monooxygenase activities, dimethyl sulfoxide seemed a likely product of dimethylsulfide oxidation by AMO (41). Analysis of reaction mixtures by GC revealed a product peak that corresponded with the retention time of authentic dimethyl sulfoxide at both 110 and 130°C. The time course of dimethyl sulfoxide production by *N. europaea* is presented in Fig. 1B. The time course of dimethyl sulfoxide production coincided with the time course of the disappearance of dimethylsulfide. Dimethyl sulfoxide accounted for an aver-

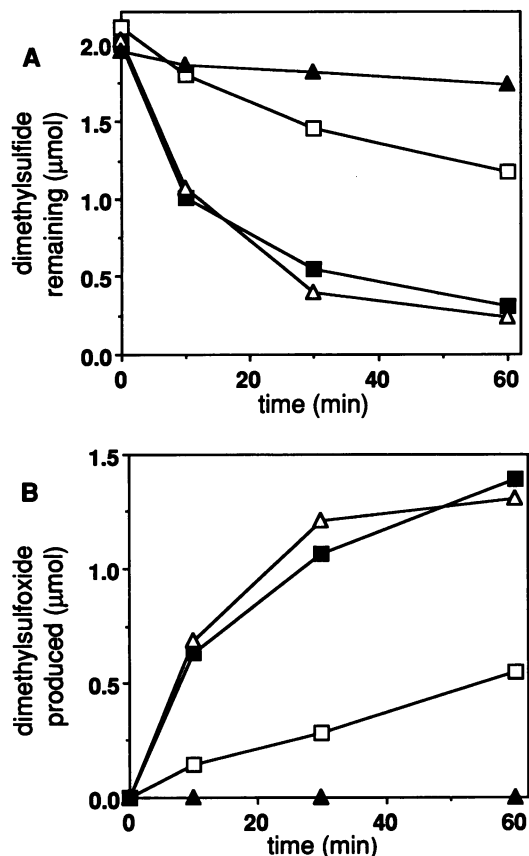



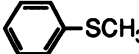


FIG. 1. Time course of dimethylsulfide oxidation and dimethyl sulfoxide formation by cells of *N. europaea*. Cell suspensions (0.1 ml, 1.4 mg of protein) were incubated with dimethylsulfide (2.0 μ mol) as described in Materials and Methods. The micromoles of dimethylsulfide remaining (A) or dimethyl sulfoxide formed (B) versus time are shown for the incubation mixtures containing C_2H_2 -treated cells and 5.0 mM $(NH_4)_2SO_4$ (▲) or active cells incubated with 0 mM (□), 2.5 mM (■), or 5.0 mM (Δ) $(NH_4)_2SO_4$. Datum points represent the averages of triplicate measurements. The relative errors for the average data varied from 1 to 12%.

age of 69% \pm 11% of the dimethylsulfide oxidized. Dimethyl sulfone, a more oxidized derivative of dimethylsulfide, was not detected as a product of cellular incubation with either dimethylsulfide (2 μ mol) or dimethyl sulfoxide (2 μ mol). If the amount of dimethylsulfide depleted which is unaccounted for as dimethyl sulfoxide had all been converted by further oxidation of dimethyl sulfoxide, enough dimethyl sulfone would have been present for detection by GC. Although formaldehyde has been observed to be a biological oxidative product of dimethylsulfide (7, 35), it was not detected as a product of dimethylsulfide oxidation by *N. europaea*.

Oxidative conversion of various thioethers to sulfoxides by *N. europaea*. It was of interest to determine whether other thioethers were substrates for AMO. Therefore, depletion of various thioethers by cell suspensions of *N. europaea* was examined (Table 2). Prior to depletion analysis, the toxicity of each thioether on the activities of AMO and HAO was determined by comparing the ammonia- and hydrazine-dependent O_2 uptake rates of cell suspensions pretreated with each thioether (1 μ mol, 60 min) with the corresponding rates for cell suspensions pretreated identically except in the

TABLE 2. Sulfide oxidation by whole cells of *Nitrosomonas europaea*^a

Compound	Structure	Amount (nmol)	Sulfide depleted ^b in 30 min (nmol)		Sulfoxide produced in 30 min (nmol)		Product detected
			No NH ₄ ⁺	5 mM NH ₄ ⁺	No NH ₄ ⁺	5 mM NH ₄ ⁺	
Methylsulfide	CH ₃ SCH ₃	2000	502±102	1331±117	293±33	1063±124	(CH ₃) ₂ SO
Ethylsulfide	CH ₃ CH ₂ SCH ₂ CH ₃	1000	200±96	757±59	— ^c	— ^c	— ^c
Tetrahydrothiophene		1000	211±61	832±125	270±23	826±42	
Thiophene		1000	310±35	882±44	— ^d	— ^d	— ^d
Methylphenylsulfide		1000	171±139	378±204	196±4	416±9	Ph-S(O)CH ₃
Allylmethylsulfide	H ₂ C=CHCH ₂ SCH ₃	1000	290±21	801±39	— ^{c,e}	— ^{c,e}	AllylS(O)CH ₃ ^{c,e}
Allylsulfide	(H ₂ C=CHCH ₂) ₂ S	1000	55±88	245±93	— ^f	— ^f	— ^f

^a The amounts of sulfide depleted and product formed were determined as described in Materials and Methods. Data represent the averages of triplicates ± the standard deviations.

^b The amount of sulfide loss from vials containing either C₂H₂-treated or boiled cells was subtracted from the amount of sulfide depleted with active cells.

^c Product peaks similar in size and retention times to the other sulfoxides were observed but were not identified or quantified because authentic sulfoxides were unavailable.

^d No product peak was observed for our GC conditions.

^e Identified by GC-MS but not quantified.

^f A small peak (~5% of the peak area of the other sulfoxides) was observed.

absence of any thioether. None of the seven thioethers we examined exhibited any irreversible effect on the activity of either AMO or HAO, under our assay conditions, except allylsulfide. Allylsulfide irreversibly inactivated the AMO activity but did not affect the HAO activity of the cell suspensions. This result indicated that allylsulfide had inhibitor properties, unlike the other thioethers examined. For five of the seven thioethers examined, 66 to 88% of the compound was depleted in the presence of ammonia by cells of *N. europaea* during a 30-min incubation (Table 2). The remaining two thioethers (methylphenylsulfide and allylsulfide) were also depleted but not to the same extent (38% for methylphenylsulfide and 24% for allylsulfide). Depletion of all the thioethers was substantially limited when incubation mixtures contained either boiled cells or cells treated with either ATU or C₂H₂. Abiotic losses of the thioethers were concluded to result primarily from losses to the stoppers. For most thioethers, abiotic losses ranged between 5 and 22% (in 30 min) of the initial amount added. However, for methylphenylsulfide, abiotic loss was higher (47% of 1 μmol in 30 min), which may account for the decreased depletion of this compound. The large standard deviations for methylphenylsulfide depletion (Table 2) were primarily a result of large variation in the background losses associated with this compound.

In addition to the product of dimethylsulfide oxidation, the products generated from incubation of the cell suspensions with either tetrahydrothiophene or methylphenylsulfide were also identified and quantified by GC. The oxidative product, generated from the incubation of *N. europaea* with either methylphenylsulfide or tetrahydrothiophene, had identical retention times at two column temperatures with authentic samples of the corresponding sulfoxides. The amount of sulfoxide formed by the cells in 30 min for each of these thioethers was comparable to the amount of thioether depleted (Table 2). This indicated that most of the thioether

depleted was accounted for by oxidation of the sulfur atom. This result was surprising because AMO has also been demonstrated to catalyze the oxidation of a variety of carbon functional groups, including the oxidation of alkanes to alcohols, alkene functions to epoxides, and the hydroxylation of benzene (16, 17, 19). However, our results indicated that the sulfoxides constituted the majority of the products of thioether oxidations. Therefore, the sulfur atom was the primary site of oxidation by AMO for the three thioethers whose sulfoxides were quantified. While the amount of dimethyl sulfoxide detected accounted for only 69% of dimethylsulfide oxidized, the amount of the sulfoxide formed by cell suspensions during incubation with either methylphenylsulfide or tetrahydrothiophene was on the average somewhat larger than the amount of the thioether depleted. The amount of methylphenylsulfoxide formed by the cell suspensions averaged 112% of the net methylphenylsulfide depleted, and the amount of tetrahydrothiophene sulfoxide formed averaged 113% of the net tetrahydrothiophene depleted. The amount of sulfoxide formed being >100% of the calculated net amount of thioether depleted may have resulted from an overestimation of the amount of background loss of sulfide from the sample vials relative to the control vials.

While the products of diethylsulfide, allylmethylsulfide, or allylsulfide oxidation were not identified or quantified, products were detected as single peaks by GC. These products were not detected in the samples containing either C₂H₂- or ATU-treated cell suspensions. During incubation of *N. europaea* with either diethylsulfide or allylmethylsulfide, the time course of formation of the products coincided with that of depletion of the parent thioether. The amount of product detected, from the incubation of cells with either diethylsulfide or allylmethylsulfide, was of sufficient quantity such that it could have accounted for the amount of thioether depleted. As for allylsulfide, only a small amount of product

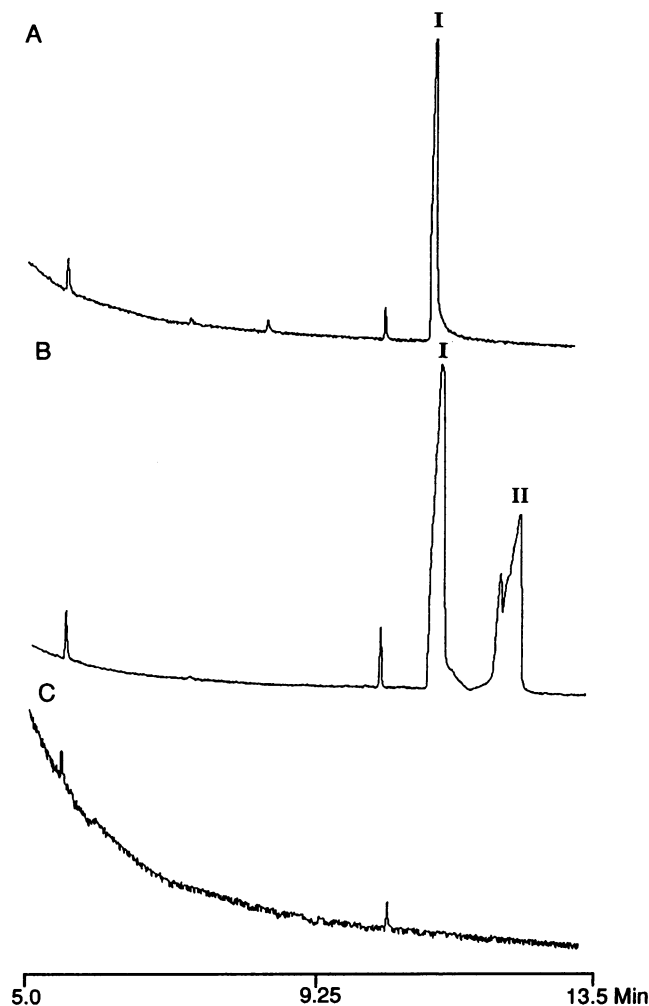


FIG. 2. Capillary GC separation and identification of allylmethylsulfoxide and allylmethylsulfone. The capillary gas chromatograms of the CH_2Cl_2 -extracted samples are shown for cells incubated in the presence of allylmethylsulfide (A), hydrogen peroxide (H_2O_2)-oxidized allylmethylsulfide (B), and cells which had been inactivated with C_2H_2 prior to incubation with allylmethylsulfide (C). The GC peaks were identified by mass spectral analyses to be allylmethylsulfoxide (I) (see Fig. 3) and allylmethylsulfone (II) (major ions at m/z 39 and 41 and a low-intensity molecular ion at m/z 120; data not presented). Acetylene-inactivated cells incubated with allylmethylsulfide did not form allylmethylsulfoxide, as shown in the chromatogram C (at increased detector sensitivity).

was detected ($\sim 5\%$ of the peak area for tetrahydrothiophene sulfoxide). Treatment of diethylsulfide, allylmethylsulfide, or allylsulfide with hydrogen peroxide produced compounds with retention times similar to those of the products generated by the cell suspensions from each of these thioethers. In the case of thiophene, no product was detected at a variety of operating temperatures with either of the two GC columns described in Materials and Methods. Thiophene sulfoxide, a predicted product, is known to be unstable (1). Although hydroxylated products of the thiophene ring were also possible products, we did not attempt to identify any of these potential products.

GC-MS identification of the product of allylmethylsulfide oxidation by *N. europaea*. The oxidative product of allylme-

thylsulfide was subjected to analysis by GC-MS for verification of the identity of the product formed by *N. europaea*. The product of allylmethylsulfide oxidation was chosen for this analysis because it was one for which an authentic sample of the putative product, allylmethylsulfoxide, was unavailable. However, analysis by GC coupled with MS could allow identification of the oxidative product of allylmethylsulfide. The capillary GC separation of the oxidative product of allylmethylsulfide, generated by *N. europaea*, is shown in Fig. 2A. This product, GC peak I, had a retention time of 11.1 min and was determined to represent allylmethylsulfoxide (molecular weight, 104) by interpretation of its mass spectral fragmentation pattern. The mass spectrum of allylmethylsulfoxide is shown in Fig. 3A. The spectrum revealed a low intensity molecular ion at m/z 104 and a base peak at m/z 41 which represents the stable allyl radical cation. The fragments formed at m/z 63 and m/z 64 are made by loss of the allyl moiety generating $\text{CH}_3\text{S}=\text{O}^+$ and $\text{CH}_3\text{S}^{16}\text{OH}^+$. These two mass fragments are typical of sulfoxides (6). Acetylene-inactivated cells incubated with allylmethylsulfide produced no peaks at the retention time assigned to allylmethylsulfoxide, indicating that AMO activity was required for the formation of allylmethylsulfoxide (Fig. 2C).

Because neither allylmethylsulfoxide nor allylmethylsulfone is commercially available and because published mass spectral fragmentation patterns were not obtained for comparison with the mass spectrum of our product, an alternative to an authentic sample of each of these compounds was generated by oxidation of allylmethylsulfide with hydrogen peroxide. The capillary GC separation of the oxidative products of allylmethylsulfide, generated with hydrogen peroxide, is shown in Fig. 2B. The peaks were identified to be allylmethylsulfoxide (peak I) and allylmethylsulfone (peak II) by analysis of their mass spectral fragmentation patterns. The retention time (11.2 min) and the mass spectrum of allylmethylsulfoxide, as obtained by chemical oxidation with hydrogen peroxide, had a similar retention time and a fragmentation pattern identical to the mass spectrum of the product generated by the cell suspensions. Because oxidation of alkenes by peracids is a slower process than oxidation of sulfenyl sulfur, the major product should be the sulfoxide, as was suggested by our GC-MS results, rather than an epoxide (30). The presence of the allylmethylsulfone, as a product of hydrogen peroxide oxidation, was not unexpected because of the excess oxidizing equivalents used in our protocol (30). However, allylmethylsulfone was not produced by cells of *N. europaea* incubated with allylmethylsulfide (compare Fig. 2A and B).

Incorporation of $^{18}\text{O}_2$ into allylmethylsulfide by cells of *N. europaea* formed ^{18}O -labeled allylmethylsulfoxide. While the use of GC-MS provided support for the identity of our products as obtained by conventional GC techniques, the use of GS-MS also enabled us to demonstrate the source of the oxygen atom incorporated into allylmethylsulfide. When cells were incubated with allylmethylsulfide in the presence of $^{18}\text{O}_2$ (97%), 82% of the sulfoxide product was labeled with ^{18}O (on the basis of the relative intensities of the molecular ions at m/z 104 and 106). A comparison of the mass spectrum of ^{18}O -labeled allylmethylsulfoxide with that of ^{16}O -labeled allylmethylsulfoxide is presented in Fig. 3. The molecular ion for allylmethylsulfoxide was shifted from m/z 104 to m/z 106, as expected for the incorporation of a single atom of oxygen derived from $^{18}\text{O}_2$ into allylmethylsulfide. The fragments at m/z 63 and 64 in the spectrum of ^{16}O -labeled allylmethylsulfoxide were also shifted to m/z 65 and 66,

confirming the presence of an ^{18}O atom in these fragment assignments. The fragments at m/z 65 and 66 in the spectrum of ^{18}O -labeled allylmethylsulfoxide were interpreted to represent $\text{CH}_3\text{S}=\text{O}^+$ and $\text{CH}_3\text{S}^{18}\text{OH}^+$. The base peaks at m/z 41 and the secondmost intense peak at m/z 39 are unshifted for each of the respective ^{16}O -labeled sulfoxides. These results established that the requirement of oxygen for sulfoxide formation was satisfied by O_2 , as expected of a reaction catalyzed by a monooxygenase.

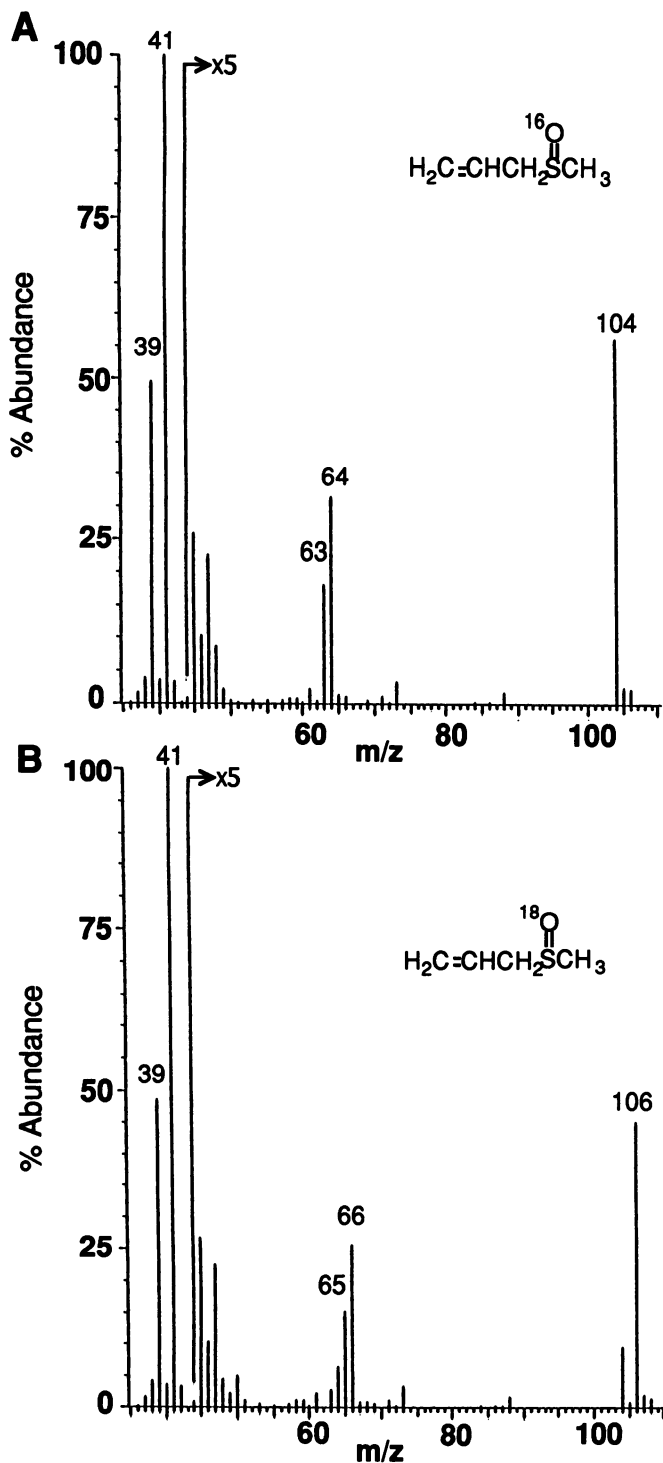


FIG. 3. Incorporation of an atom of ^{18}O from $^{18}\text{O}_2$ into allylmethylsulfoxide by cells of *N. europaea*. Mass spectra are shown for allylmethylsulfoxide formed during incubations which were conducted in the presence of 20% O_2 added as an overpressure of either 100% $^{16}\text{O}_2$ (A) or 97.7% $^{18}\text{O}_2$ (B). The mass spectral fragmentation pattern presented in panel A corresponds to the GC peak (I) in Fig. 2A. ^{16}O -labeled allylmethylsulfoxide had major ions at m/z 39, 41, 45, 47, 63, and 64 and a molecular ion at m/z 104 (A). A GC peak which was similar in magnitude and which was eluted at the same retention time as peak I was observed when the incubation was conducted in the presence of $^{18}\text{O}_2$ (data not presented). The spectral fragmentation pattern of ^{18}O -labeled allylmethylsulfoxide had major ions at m/z 39, 41, 45, 47, 65, and 66 and a molecular ion at m/z 106 (B). The intensities of the fragments have been enhanced fivefold for mass fragments greater than mass 44.

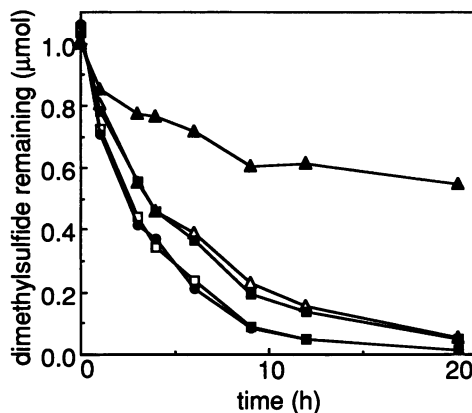


FIG. 4. Depletion of dimethylsulfide by an oceanic ammonia-oxidizing nitrifier, *N. oceanus*. Cell suspensions of *N. oceanus* (0.05 ml, 0.7 mg of protein) were incubated with dimethylsulfide (1 μmol) as described in Materials and Methods. The micromoles of dimethylsulfide remaining versus time are shown for incubation mixtures containing C_2H_2 -treated cells and 2.5 mM $(\text{NH}_4)_2\text{SO}_4$ (\blacktriangle) or active cells incubated with 0 mM (\triangle), 0.025 mM (\blacksquare), 1.2 mM (\square), or 2.5 mM (\bullet) $(\text{NH}_4)_2\text{SO}_4$. Data represent averages of duplicate samples. The relative errors ranged from 1 to 12%.

Although the incorporation of $^{18}\text{O}_2$ into allylmethylsulfoxide was less than the theoretical value of 97%, it was consistent with the isotope ratio, as determined by the gas analyzer, averaged over the time course of the experiment. The headspace gas composition of the vials was analyzed before and after the incubations by using a residual gas analyzer. The amounts of $^{18}\text{O}_2$ and $^{16}\text{O}_2$ in the vials were recorded as the partial pressures of mass 36 and mass 32, respectively. The fraction of $^{18}\text{O}_2$ in the headspace of the reaction vial changed from 96% $^{18}\text{O}_2$ at the start of the experiment to 77% at the end (percent expressed relative to the amount of $^{18}\text{O}_2$ plus $^{16}\text{O}_2$ present). The headspace composition, as measured by the residual gas analyzer, was consistent with the appearance of ^{16}O -labeled allylmethylsulfoxide (molecular ion at m/z 104) with a relative intensity of 17% in the mass spectrum of ^{18}O -labeled allylmethylsulfoxide (Fig. 3B). The mass spectrum of ^{16}O -labeled allylmethylsulfoxide had of a 5% sulfoxide component with a molecular ion at m/z 106, and the spectrum of ^{18}O -labeled allylmethylsulfoxide had a 5% component at m/z 108 due to the 4.4% isotopic abundance of ^{34}S .

Oxidation of dimethylsulfide to dimethylsulfoxide by the marine nitrifier *N. oceanus*. Because of the importance of

dimethylsulfide in the oceanic sulfur cycle (36), depletion of dimethylsulfide by an oceanic nitrifier was investigated. The time course of the oxidation of dimethylsulfide by *N. oceanus* is presented in Fig. 4. The oxidative product was identified by GC as dimethyl sulfoxide. Dimethylsulfide depletion and dimethyl sulfoxide formation were prevented by treating *N. oceanus* with C_2H_2 . Treatment of cell suspensions of *N. oceanus* with ATU (100 μM) did not significantly limit depletion of dimethylsulfide as it had for *N. europaea*. Differences in susceptibility to nitrification inhibitors between *N. europaea* and *N. oceanus* have been reported previously (22). However, as our results indicate, acetylene did prevent AMO-catalyzed oxidations in both *N. europaea* and *N. oceanus* (22). Depletion of dimethylsulfide by *N. oceanus* occurred more slowly than depletion by *N. europaea*. Cell suspensions of *N. oceanus* (0.7 mg of protein) consumed 1 μmol of dimethylsulfide in 20 h, while cell suspensions of *N. europaea* (1.4 mg of protein) consumed 1.3 μmol in 0.5 h. The ammonia-oxidizing activities of these cell suspensions, as determined by the rates of ammonia-dependent O_2 uptake, were similar ($\sim 30 \mu mol$ of O_2 consumed per h per mg of protein for *N. oceanus* and $\sim 40 \mu mol$ of O_2 consumed per h per mg of protein for *N. europaea*). Another feature of dimethylsulfide depletion by *N. oceanus*, which was distinct from the time course of dimethylsulfide depletion by *N. europaea*, was the high rate of dimethylsulfide consumption in the absence of ammonia relative to the rate in the presence of ammonia. The rate of dimethylsulfide consumption by *N. oceanus* was only slightly slower in the absence of ammonia than in the presence of ammonia (Fig. 4). On the other hand, incubation of *N. europaea* for 20 h with dimethylsulfide (1 μmol) in the absence of ammonia did not result in complete depletion of dimethylsulfide as it did for *N. oceanus*. Both of these results suggested that high levels of an endogenous reductant may be present in *N. oceanus*. It is not unusual for bacteria such as *N. oceanus*, which are presumably adapted to the oligotrophic conditions of seawater, to have high levels of an endogenous reductant (29).

DISCUSSION

We investigated the properties of dimethylsulfide inhibition of ammonia oxidation by *N. europaea* and conclude that the inhibition is a result of the oxidation of dimethylsulfide by AMO. Depletion of several other thioethers by cells of *N. europaea* was also demonstrated (Table 2). The products formed by oxidation of three different thioethers were identified to be the corresponding sulfoxides. The following evidence supports the idea that AMO catalyzed the oxidation of the thioethers. (i) Both a reductant source and O_2 , which are required for the activity of AMO, were also required for depletion of dimethylsulfide and formation of dimethyl sulfoxide. (ii) The AMO-specific inhibitors C_2H_2 and ATU prevented depletion by *N. europaea* of each of the sulfides listed in Table 2. (iii) AMO-specific inhibitors also prevented formation of the sulfoxide products. (iv) Mass spectral analysis demonstrated that a single atom of ^{18}O from $^{18}O_2$ was incorporated into allylmethylsulfide, producing ^{18}O -labeled allylmethylsulfoxide.

AMO can catalyze the oxidation of N-H bonds of ammonia, aryl C-H (17), alkyl C-H (16, 18), and C=C (19, 20, 33) and can oxidatively cleave C-O-C bonds (12a). The results of this work show that AMO can also catalyze the oxidation of thioethers to sulfoxides. The amount of thioether depleted by the cell suspensions in the case of dimethylsulfide,

methylphenylsulfide, and tetrahydrothiophene was predominantly accounted for as the sulfoxides. This indicated that AMO preferentially oxidized the sulfur atom over the alkane or phenyl moiety. Because the amount of dimethylsulfoxide formed was small (69%) in relationship to the amount of dimethylsulfide depleted, we cannot exclude the possibility that a small amount of some other oxidative product(s) may have been formed. However, dimethylsulfone, a potential oxidative product, was not detected. The inability of cell suspensions to further oxidize sulfoxides to sulfones was also supported by results obtained by GC-MS which showed that allylmethylsulfone was not a product of allylmethylsulfide oxidation by *N. europaea*. On the basis of the magnitude of the GC peak identified as allylmethylsulfoxide, oxidation of the sulfur atom of allylmethylsulfide was preferred over oxidation of the alkene moiety. While our data cannot rigorously exclude the possibility that other oxidative products are formed, the data clearly support the conclusion that the majority of the products formed are sulfoxides. The oxidation of the sulfur atom of these sulfides in preference to the other carbon functional groups available for oxidation might be expected in light of the ease of oxidation of sulphenyl sulfur over oxidation of carbon atoms (30).

Sulfur is generally susceptible to oxidation, and the oxidation of a sulfide to a sulfoxide is a common reaction for a microbial or mammalian monooxygenase (11). Most often the oxidation is attributed to a monooxygenase activity due to a requirement for O_2 . Microsomal fractions from liver and two purified enzyme systems, cytochrome P-450 monooxygenase and dopamine β -hydroxylase, have been shown to oxidize sulfides to the sulfoxides (28, 41). Cell suspensions and cellular extracts of both bacteria and fungi have been shown to oxidize sulfides to sulfoxides, and in some cases sulfone is also formed (12, 31, 39). Although cell suspensions of *N. europaea* oxidized sulfides to sulfoxides, they did not oxidize sulfides to the oxidative state of sulfones. The lack of sulfone as a potential product is consistent with the inert behavior of dimethyl sulfoxide on the ammonia metabolism of *N. europaea* (3). Dimethyl sulfoxide has been used as an alternate solvent for many water-insoluble organic compounds used in the study of ammonia-oxidizing bacteria (3, 15).

Global mass balances for sulfur indicate that dimethylsulfide is an important volatile biogenic compound involved in the transfer of sulfur from the ocean to the atmosphere (36). The main source of dimethylsulfide in the ocean is phytoplankton. Microbial consumption of dimethylsulfide is an important aspect of dimethylsulfide biogeochemistry in seawater. Although oceanic microbial consumption of dimethylsulfide has been reported (24, 25), the aerobic metabolism of dimethylsulfide by a pure culture of marine bacteria has not yet been documented (36). Methylotrophic organisms and/or chemolithotrophs have been speculated to contribute to depletion of oceanic dimethylsulfide because depletion was sensitive to a common inhibitor of C_1 metabolism, chloroform (24, 25). Therefore, we investigated the ability of a marine nitrifier to consume dimethylsulfide. Our results indicated that *N. oceanus* oxidized dimethylsulfide (Fig. 4) and that the product formed was dimethyl sulfoxide. Because the oxidation of dimethylsulfide and formation of dimethyl sulfoxide were prevented by C_2H_2 , the oxidation most likely involved AMO. This result raises the possibility that the process of co-oxidation by marine nitrifiers may contribute to the oceanic sulfur cycle.

In summary, our results suggested that dimethylsulfide in particular and thioethers in general act as substrates for

AMO. Depletion of dimethylsulfide by two ammonia oxidizers, *N. europaea* and *N. oceanus*, was prevented by treating cell suspensions with C_2H_2 , a specific inactivator of AMO. For three of the thioethers we examined, the majority of the oxidative product formed by cells of *N. europaea* was established to be the corresponding sulfoxide. The incorporation of a single atom of ^{18}O from $^{18}O_2$ into allylmethylsulfide to form ^{18}O -labeled allylmethylsulfoxide by cells of *N. europaea* also supported our conclusions of the catalytic involvement of AMO in the oxidation. The demonstration that *N. europaea* can oxidize a variety of thioethers extends the known substrate range of AMO to specifically include the sulfur atom as a potential site of oxidation in addition to the already established sites, the carbon and nitrogen atoms.

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