Oxidation of Methyl Fluoride and Dimethyl Ether by Ammonia Monooxygenase in *Nitrosomonas europaea*

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Methyl fluoride and dimethyl ether were previously identified as inhibitors of ammonia oxidation and N_2O production in autotrophic nitrifying bacteria. We demonstrate that methyl fluoride and dimethyl ether are substrates for ammonia monooxygenase and are converted to formaldehyde and a mixture of methanol and formaldehyde, respectively.

Specific inhibitors are important tools in the study of bacterial roles in nutrient cycles (11). For example, acetylene is used for estimating both nitrogen-fixing activity and denitrification activity (5). Recently methyl fluoride (MF) (CH_3F) and dimethyl ether (DME) [$(CH_3)_2O$] have been described as specific inhibitors of methane oxidation (12, 13) and nitrification (10). Complete inhibition (>95%) of ammonia oxidation is achieved with 10% (vol/vol) MF and 25% (vol/vol) DME (10). The attraction of MF and DME is that these compounds can be used to investigate N_2O production by nitrifying bacteria, because, unlike acetylene, these compounds do not affect heterotrophic denitrification (12).

The site of action of MF and DME as inhibitors of ammonia oxidation was suggested to be ammonia monooxygenase (AMO) (10). This enzyme initiates the oxidation of ammonia. Like the related methane monooxygenase, AMO has a relaxed substrate specificity (1, 2, 6, 7, 14–16). This study reveals that the mechanism of inhibition of AMO by MF and DME involves these compounds acting as substrates for AMO.

Cells of *Nitrosomonas europaea* (ATCC 19718) were grown, harvested, and resuspended as described previously (6, 14). The oxidation of MF and DME was determined with incubations conducted in glass serum vials (10 ml) sealed with Teflon-lined septa. The reaction mixtures consisted of 50 mM sodium phosphate buffer (pH 7.8) with 2 mM $MgSO_4$ and 5 mM $(NH_4)_2SO_4$ (0.9 ml). DME and MF were added as pure gases. The reactions were initiated by adding cell suspension (100 μl [approximately 2 mg of total protein]). The vials were placed in a heated (30°C), reciprocating water bath. The reactions were stopped after 60 min by adding allylthiourea (ATU) (100 μM final concentration), a reversible inhibitor of AMO. Samples were then analyzed for methanol and formaldehyde. The level of methanol was determined by gas chromatography with flame ionization detection (6). Formaldehyde was detected enzymatically (14). The level of nitrite was determined colorimetrically (4). Protein concentrations were determined by the biuret method (3), and bovine serum albumin was used as a standard.

Incubations with MF resulted in formaldehyde generation and inhibition of ammonia oxidation (Fig. 1). No formaldehyde or nitrite was formed in the presence of ATU (data not shown). The maximal inhibition of nitrite production we achieved (85%) occurred with 90 μmol of MF. The largest amount of formaldehyde was generated in the presence of 20

μmol of MF, although there was only a 20% difference in formaldehyde production between cells incubated with 20 μmol of MF and those incubated with 90 μmol of MF. Previous studies demonstrated complete inhibition of ammonia oxidation with 10% (vol/vol) MF (10). This is equivalent to the addition of 40 μmol of MF in our experiments.

Cells incubated with DME generated both formaldehyde and methanol (Fig. 2). The largest amount of organic products was observed in the presence of 11 μmol of DME, although 45 μmol of DME was required to inhibit nitrite production by >95%. Notably, both formaldehyde and methanol were still produced in the presence of these high concentrations of DME. No methanol, formaldehyde, or nitrite formation was observed in the presence of ATU (data not shown). Our results indicate DME is a more effective inhibitor of ammonia oxidation than previously reported, because earlier studies demonstrated 25% (vol/vol) DME was required to fully inhibit ammonia oxidation. This is equivalent to approximately 100 μmol of DME in our experimental system.

The data presented in Fig. 1 and 2 are representative of several experiments and provide clear evidence that MF and DME are substrates for AMO. The quantities of products obtained in individual experiments varied considerably. For example, over the course of four individual experiments with each compound we observed a 2.5-fold variation in the maximal quantity of formaldehyde generated per milligram of protein. This level of variability is often observed in cooxidation experiments (14), and this is primarily a function of variation in the specific ammonia-oxidizing activity of individual cell preparations. In contrast to the variation in the absolute quantities of products obtained, the profiles of product generation from MF and DME versus substrate concentration (Fig. 1 and 2) were essentially identical in all experiments. This unusual profile is a direct consequence of the cometabolic oxidation of these compounds and is dictated by the affinities of AMO for each compound, relative to ammonia. For example, maximal MF and DME oxidation occurred at lower concentrations than were required to fully inhibit ammonia-dependent nitrite production. Alternative substrates (e.g., MF and DME) compete with ammonia for oxidation by AMO and decrease the normal rate of supply of reductant to AMO. Accordingly, MF and DME oxidation is greatest while ammonia oxidation is also occurring and can provide reductant for AMO activity. In the absence of ammonia, DME and MF oxidation is supported by reductant derived from endogenous respiration.

DME oxidation by AMO would be expected to produce equimolar concentrations of formaldehyde and methanol.

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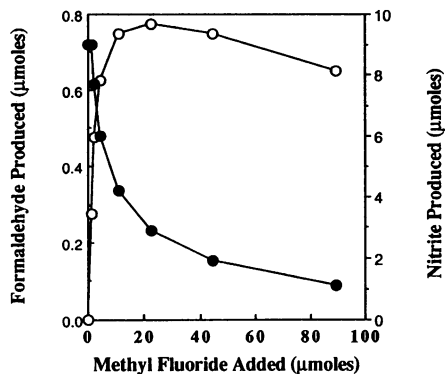
FIG. 1. Effect of MF concentration on formaldehyde production and inhibition of ammonia oxidation by *N. europaea*. Cells were incubated with MF for 60 min, and formaldehyde and nitrite levels were determined as described in the text. ●, micromoles of nitrite detected; ○, micromoles of formaldehyde detected.

However, this product distribution will not be observed if further enzymatic oxidation of methanol to formaldehyde occurs (13, 16). Our results show considerable deviation from a 1:1 stoichiometry of organic products from DME oxidation. Therefore, secondary reactions may have occurred with one or both products. Previous studies showed that methanol is
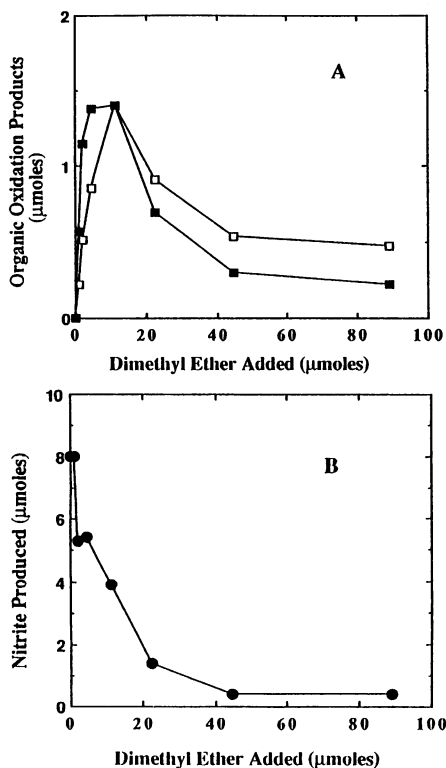

FIG. 2. Effect of DME concentration on formaldehyde and methanol production and inhibition of ammonia oxidation by *N. europaea*. Cells were incubated with DME for 60 min, and formaldehyde, methanol, and nitrite levels were determined as described in the text. (A) Micromoles of formaldehyde (■) and methanol (□) detected. (B) Micromoles of nitrite (●) detected.

oxidized to formaldehyde by AMO (16). We observed that more than 80% of 1 mM exogenous methanol was depleted after incubation with cells and 10 mM NH_4^+ for 60 min. Formaldehyde production accounted for as much as half the concentration of the methanol depleted. No methanol depletion or formaldehyde production occurred in the presence of ATU. These data suggest that AMO activity is required for the production of formaldehyde from methanol. The stability of formaldehyde was also examined. The amount of formaldehyde consumed during the 60-min incubation (125 to 250 nmol) was not strongly influenced by the amount of formaldehyde added (250 to 1,250 nmol). Formaldehyde depletion was unaffected by ATU, and in the absence of cells, 97 to 100% of the added formaldehyde remained. The depletion of formaldehyde may be due partly to nonenzymatic factors because 50 to 100 nmol of formaldehyde was consumed in samples containing boiled cells.

Our results establish that MF and DME are substrates for AMO, and we suggest that this is their likely mode of action as inhibitors of ammonia oxidation. The oxidation of MF to formaldehyde is not unexpected, since a similar reaction occurs with other monohalogenated methanes (7, 14). DME oxidation, however, is the first example of an *O*-dealkylation reaction catalyzed by AMO. This reaction joins the previously established hydroxylation (6), epoxidation (6), and dehalogenation (14) reactions catalyzed by AMO. The inhibitory effects of these compounds are also fully reversible after washing cells to remove the inhibitor, and this further supports our simple explanation for the effects of these compounds. Because the products of these compounds may be either further metabolized, absorbed, or excreted by *N. europaea*, it is not clear if additional effects should be attributed to these compounds in longer-term experiments than those described in this study.

The desirable inhibitory effects of MF and DME on nitrification could potentially be achieved by any highly soluble alternative AMO substrate. The alternative approach to specific substrate inhibitors of AMO such as MF or DME is the use of mechanism-based inactivators of AMO. These include *n*-alkynes (6, 8) and allylsulfide (9). Many of these compounds are gaseous and are not transformed significantly by AMO. Considerably lower concentrations of these compounds are required to irreversibly inhibit ammonia oxidation compared with MF and DME. It is not known whether these compounds (other than acetylene) inhibit N_2O reductase and denitrification. However, if unreactive, these compounds would retain the same advantages of MF and DME and would be useful inhibitors for investigating nitrifier-dependent N_2O production.

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