COMPETITIVE INHIBITION OF AMMONIA OXIDATION IN *NITROSOMONAS EUROPAEA* BY METHANE, CARBON MONOXIDE OR METHANOL

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1. Introduction

Ammonia oxidation by *Nitrosomonas europaea* and methane oxidation by methane-utilizing bacteria seem to have a striking similarity as pointed out by the workers of the latter organisms [1,2]. Both oxidations presumably start with hydroxylation of the substrates: $NH_3 \longrightarrow NH_2OH \longrightarrow (NOH) \longrightarrow NO_2^-$ [3] and $CH_4 \longrightarrow CH_3OH \longrightarrow HCHO \longrightarrow HCOOH \longrightarrow CO_2$ [1] and are sensitive to similar inhibitors [1,4]. In addition the oxidation of methane is competitively inhibited by ammonia [1,2].

Although ammonia-oxidizing cell-free systems have been successfully prepared [5,6], it has been difficult to obtain cell-free extracts of methane-utilizing bacteria which will oxidize methane reproducibly [1]. The extracts, however, showed methane-stimulated NADH oxidation [7] and CO-stimulated NADH oxidation [8] activities. The stoichiometry of these oxidations confirmed the involvement of a monooxygenase (hydroxylase) [2,9]: $CH_4 + O_2 + NADH + H^+ \longrightarrow CH_3OH + NAD^+ + H_2O$ or $CO + O_2 + NADH + H^+ \longrightarrow CO_2 + NAD^+ + H_2O$.

This paper reports the competitive inhibition of ammonia oxidation by methane, carbon monoxide or methanol and the presence of an ammonia-dependent NADH oxidation activity in *Nitrosomonas* extracts. Requirement for NADH or hydroxylamine as electron donor in ammonia oxidation is also demonstrated. The mechanism of ammonia oxidation is compared to that of methane oxidation.

2. Materials and methods

Growth of *Nitrosomonas europaea* and preparations of ammonia-oxidizing cell-free extracts (with 20 mg/ml bovine serum albumin) were described previously [6,10]. Oxidation of ammonia was followed by a polarographic measurement of oxygen disappearance in a Gilson Oxygraph at 25°C (1.5 ml reaction mixture in 0.1 M potassium phosphate of pH 7.5) as described [6,10]. The particulate or membrane fraction was prepared by passage of 4 ml extracts through a column (2 X 30 cm) of Sepharose 6B (Pharmacia) in 0.1 M potassium phosphate (pH 7.5) and elution with the same buffer. The brown fraction (6 ml) eluted within the void volume was used for the experiments. Nitrite was determined as previously described [11].

3. Results and discussion

Ammonia oxidation by *N. europaea* cells was inhibited by methane, carbon monoxide and methanol as shown in table 1. The inhibition was stronger with the lower concentration of ammonia. Methane or CO oxidation by methane-oxidizing bacteria is competitively inhibited by ammonia [2]. Although the inhibition in table 1 largely disappeared at a high ammonia concentration suggesting competitive inhibition, the rate-ammonia concentration plot became sigmoidal in the presence of CH_4, CO or CH_3OH indicating a complex phenomenon possibly involving transport of the substrate.

Ammonia oxidation by cell-free extracts of *N. europaea* was inhibited competitively by methane, carbon monoxide or methanol as shown in figs.1–3. The K_i values obtained for CH_4 and CO, 50 μM and 3 μM, compare favourably with the K_m values of 15 μM CH_4 and 2.7 μM CO for the methane-oxidizing

Table 1
Inhibition by methane, carbon monoxide and methanol of ammonia oxidation by *Nitrosomonas* cells

Additions	Activity (μmol O_2/min/mg wet cells)	
	1 mM $(NH_4)_2SO_4$	0.33 mM $(NH_4)_2SO_4$
None	100	50
CH_4 (0.5 mM)	90	19
CO (20 μM)	80	13
CH_3OH (1.7 μM)	90	16

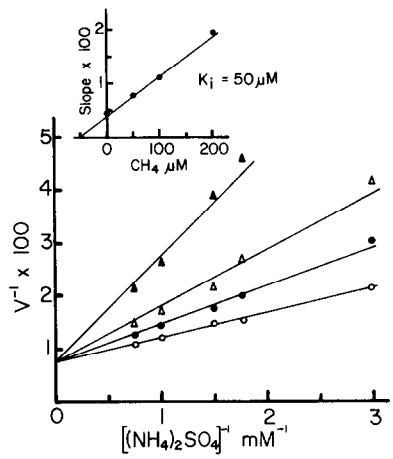

Fig.1. Competitive inhibition of ammonia oxidation by methane in extracts of *Nitrosomonas*. V, nmol O_2 consumed/min/0.5 ml extract.

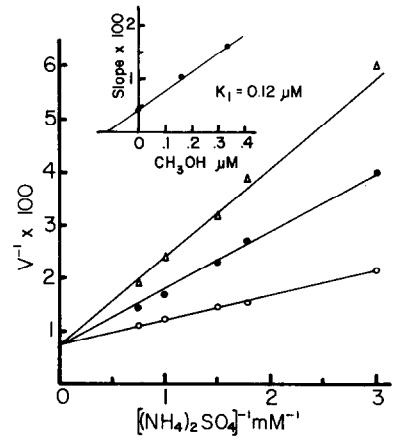

Fig.3. Competitive inhibition of ammonia oxidation by methanol in extracts of *Nitrosomonas*. V, nmol O_2 consumed/min/0.5 ml extract.

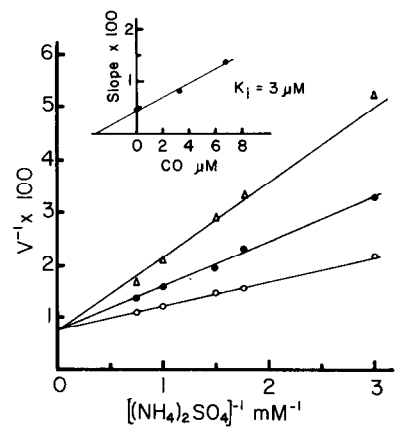

Fig.2. Competitive inhibition of ammonia oxidation by carbon monoxide in extracts of *Nitrosomonas*. V, nmol O_2 consumed/min/0.5 ml extract.

Table 2
NADH oxidation by a particulate fraction of *Nitrosomonas* extracts

Additions	NADH oxidation (nmol NADH/min/mg protein)
None	1.2
$(NH_4)_2SO_4$ (2 mM)	4.5
CH_4 (1 mM)	5.5
CO (0.4 mM)	6.5
CH_3OH (50 μM)	4.5

NADH oxidation was measured spectrophotometrically at 340 nm. NADH concentration: 0.1 mM, pH 7.5, in 0.1 M potassium phosphate.

Table 3
Effect of NADH, NH_2OH and bovine serum albumin on the oxidation of ammonia by *Nitrosomonas* extracts

Additions	O_2 consumed (nmol)	Nitrite formed (nmol)
$(NH_4)_2SO_4$	0	0
NADH	20	10
$(NH_4)_2SO_4$ + NADH	260	160
NH_2OH	50	40
$(NH_4)_2SO_4$ + NH_2OH	280	180
$(NH_4)_2SO_4$ + Albumin	260	165

The reaction was carried out for 7 min in a total volume of 1.5 ml in 0.1 M potassium phosphate (pH 7.5) containing 0.5 ml cell-free extract (1.5 mg protein) prepared without bovine serum albumin. Additions: ammonium sulfate, 2.5 μmol; hydroxylamine, 50 nmol; NADH, 50 nmol; bovine serum albumin, 30 mg. The endogenous (without additions) values of 10 nmol O_2 consumed and 10 nmol nitrite found were substracted from the experimental results to obtain the numbers shown in the table.

bacteria [2]. Neither CH_4, CO nor CH_3OH was oxidized by *Nitrosomonas* cells or extracts.

The extracts of *N. europaea* had an ammonia-stimulated NADH oxidase activity. The activity resided in the particulate or membrane fraction of the extracts as was the case with the methane-dependent NADH oxidation by methane-oxidizing bacteria [2,7]. Ammonia could be replaced by methane, carbon monoxide or methanol as shown in table 2. There was a slow disappearance of NADH under anaerobic conditions, but the rate was unaffected by the addition of ammonia. The ammonia-dependent NADH oxidation rate was only one tenth of that reported for the methane- or carbon monoxide-dependent NADH oxidation by methane-oxidizing bacteria [7,8].

The role of NADH in the ammonia oxidation seems to be catalytic rather than stoichiometric as in methane oxidation [2,7,8]. The catalytic activation of *Nitrosomonas* extracts by NADH or hydroxylamine is shown in table 3. For these experiments the extracts were prepared without bovine serum albumin. These extracts were now similar to the cell-free extracts of methane bacteria in requiring the presence of NADH for the oxidation of substrate. Hydroxylamine, a presumed intermediate of ammonia oxidation, could replace NADH. Hydroxylamine eliminated an initial lag phase of ammonia oxidation by resting *Nitrosomonas* cells [12] and activated the spheroplasts for ammonia oxidation [11]. With the addition of bovine serum albumin the extracts oxidized ammonia without NADH (table 3). Spermine or $MgCl_2$ could replace albumin as reported previously [6]. Ascorbate, which was effective as electron donor in methane oxidation [13], did not replace NADH in ammonia oxidation.

These results confirm and extend the concept that the initial stage of ammonia oxidation is very similar to that of methane oxidation. Methane or CO, the oxidizable substrate for methane-oxidizing system, and methanol, the first intermediate in methane oxidation, all seem to compete for the ammonia-binding site of *Nitrosomonas* (figs.1–3) and can replace ammonia in the ammonia-stimulated NADH oxidation (table 2). Since the undissociated form of ammonia, NH_3, rather than ammonium ion, NH_4^+, seems to be the substrate for oxidation by *Nitrosomonas* [10], the structural similarity with these one carbon compounds is obvious. The catalytic rather than stoichiometric role of NADH in ammonia oxidation is possibly related to the intactness of the system in the extracts where, unlike methane oxidation, complete oxidation of ammonia to nitrite is achieved thus generating necessary reducing power for ammonia activation during hydroxylamine oxidation [3,11]. Replacement of NADH with hydroxylamine supports the above conclusion. In the presence of bovine serum albumin the system becomes essentially similar to that of intact cells oxidizing ammonia without addition of NADH or hydroxylamine. Since NADH is generated during methane oxidation in at least one reaction, formic dehydrogenase [1], while no such reaction is known in ammonia oxidation, NADH is probably not a natural electron donor for the ammonia activation once the ammonia oxidation to nitrite is initiated.

Acknowledgement

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