Oxidation of Ammonia by Methane-oxidizing Bacteria and the Effects of Ammonia on Methane Oxidation

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INTRODUCTION

Certain similarities between methane-oxidizing bacteria and ammonia-oxidizing bacteria suggest an evolutionary relationship (Wilkinson, 1971; Quayle, 1972), but the interactions of methane-oxidizing bacteria with ammonia have only briefly been described. Hutton & Zobell (1949) and Whittenbury, Phillips & Wilkinson (1970) observed that methane-oxidizing bacteria produced nitrite from ammonia, and the latter workers also noted the competitive inhibition of methane utilization by ammonia. Recently Ferenci, Strøm & Quayle (1975) showed that ammonia could act as a competitive inhibitor for both methane and carbon monoxide oxidation in *Pseudomonas methanica*. In addition Hubley, Thomson & Wilkinson (1975) reported that the specificity of a number of inhibitors of ammonia oxidation in *Nitrosomonas*, as described by Hooper & Terry (1973), followed a similar pattern when inhibiting methane oxidation in *Methylosinus trichosporium*.

This work attempts to describe more precisely the nature of the interactions of ammonia with methane-oxidizing bacteria.

METHODS

Organisms and growth. *Methylosinus trichosporium* OB3B and *Methylomonas albus* BG8 (Whittenbury *et al.*, 1970) were grown on methane, harvested in the exponential phase of growth and suspensions were prepared as described by Hubley, Mitton & Wilkinson (1974).

Experiments with bacterial suspensions. Respiration studies used an oxygen electrode as described by Hubley *et al.* (1974). Routinely, 4.7 mg dry wt bacteria were suspended in 2.7 ml 20 mM- $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer and oxygen uptake was recorded for 10 min at 30 °C. For other incubations, a suspension of 47 mg dry wt bacteria in 5 ml 20 mM-phosphate buffer in a 30 ml round-bottomed Quickfit flask fitted with a rubber Suba-seal stopper was agitated on a Griffin and Tatlock Microid wrist-action shaker at 30 °C.

Gas determination. Methane and O_2 were measured using a Pye series 104 chromatograph with a katharometer detector and a 2.8 m silica gel column at 140 °C and N_2 as carrier gas. Gas samples (0.2 ml) were taken from the incubation flasks for analysis. Nitrous oxide was measured using the above system but at a temperature of 50 °C (to ensure separation from CO_2).

Nitrite and ammonia determinations. Samples (0.2 ml) for determining nitrite or ammonia were taken from the incubation flasks or from the oxygen electrode vessel and added to 0.8 ml phosphate buffer in a 1.5 ml microcentrifuge tube. Bacteria were sedimented by

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centrifuging for 30 s in a Quickfit Micro-centrifuge and 0.5 ml of the supernatant fluid was taken to determine either nitrite using the 'diazotization' method described by Nicholas & Nason (1957), or ammonia using the method of Van Slyke & Hiller (1933).

Materials. Methane, nitrogen, nitrous oxide and nitric oxide were obtained from The British Oxygen Co., Special Gases, London. Silica gel (80 to 100 mesh) was from Phase Separations, Queensferry, Clwyd.

Throughout this work 'ammonia' indicates the total concentration of $\text{NH}_3 + \text{NH}_4^+$.

RESULTS

Ammonia oxidation

A washed suspension of *M. trichosporium* in 20 mM-phosphate buffer pH 6.8 took up oxygen, in the presence of 10 mM-ammonia, at a rate of $4.1 \text{ nmol min}^{-1} (\text{mg dry wt bacteria})^{-1}$. This compares with 1.3 and 73 for the endogenous rate and the rate in the presence of 0.6 mM-methane respectively. Associated with the oxidation of ammonia was a stoichiometric disappearance of ammonia and appearance of nitrite, both at rates which were linear over at least 100 min. If the oxygen uptake in the presence of ammonia was corrected by subtracting the endogenous level, the results were consistent with the stoichiometry:

This is the same as that established for ammonia-oxidizing bacteria (Hofman & Lees, 1953). Hydroxylamine was also oxidized to nitrite by methane-oxidizing bacteria, but at a higher rate than ammonia.

Effect of various compounds on ammonia oxidation. Both ammonia and hydroxylamine oxidation were subject to product inhibition by nitrite; nitrite production tailed off when the concentration of nitrite increased to 3 to 4 mM. Furthermore, if 3 mM-nitrite was initially included in the incubation mixture, then nitrite production from ammonia or hydroxylamine was inhibited by more than 90%.

Formate stimulates the rate of oxidation of a number of substrates unable to support growth in methylobacteria, i.e. carbon monoxide (Ferenci, 1974), ethane (Thomson, 1974) and ethylene (Hubley, 1975). Formate had a similar effect on ammonia oxidation but in addition produced another stimulatory effect. The rate of nitrite production from ammonia in stationary phase bacteria was considerably slower than in exponential phase bacteria and also tended to decline at nitrite concentrations below those shown to be inhibitory. If formate was added at this stage of the incubation, nitrite production recommenced until inhibitory levels were reached.

Several other substrates (at 10 mM) were tested for their ability to stimulate ammonia oxidation. The rates of nitrite production [$\text{nmol min}^{-1} (\text{mg dry wt bacteria})^{-1}$] from 10 mM-ammonia were: formate, 17.5; formaldehyde, 10.3; 3-hydroxybutyrate, 9.6; pyruvate, 7.1; acetate, 6.5; and methanol, 6.0. These compare with a value of 5.8 for ammonia alone. No stimulation of the rate of nitrite production from hydroxylamine was detected for any of these substrates.

Nitrite removal. Suspensions of bacteria grown with nitrate as the sole nitrogen source removed nitrite from the suspending medium when it was added initially and also after it had been produced from ammonia. The activity was stimulated by formate. This removal of nitrite is probably due to a functioning assimilatory nitrate pathway (see, for example, Payne, 1973). Probably related to this pathway was the occasional detection of nitrous oxide in the atmosphere over the incubation mixtures. The production of N_2O from nitrite

has been previously recorded for *Nitrosomonas* and a number of non-denitrifying heterotrophic bacteria and fungi (Yoshida & Alexander, 1970; Ritchie & Nicholas, 1972).

Effect of pH on ammonia oxidation and inhibition. Varying the pH had a far greater effect on ammonia oxidation than on methane oxidation by *M. trichosporium*; the rate of ammonia oxidation increased considerably with pH. Inhibition of methane oxidation by ammonia increased as the pH increased. Thus, at pH 6.0 the rate of oxidation of 10 mM-ammonia, as measured by oxygen uptake, was $0.8 \text{ nmol min}^{-1} (\text{mg dry wt bacteria})^{-1}$. This concentration of ammonia also inhibited oxidation of 0.5 mM-methane by 6%. At pH 8.0 the rate of ammonia oxidation was $6.2 \text{ nmol min}^{-1} (\text{mg dry wt})^{-1}$ and methane oxidation was inhibited by 59%.

A possible explanation for the pH effects involving ammonia is that the active species is NH_3 and not, as has been suggested, NH_4^+ (Whittenbury *et al.*, 1970). The proportion of NH_3 to NH_4^+ will decrease logarithmically with pH according to the Henderson-Hasselbalch relationship. Work by Suzuki, Dular & Kwok (1974) has suggested that NH_3 and not NH_4^+ is the substrate for *Nitrosomonas*. These authors showed that an apparently large variation in K_m for ammonia with pH resolved to considerably closer values when calculated in terms of NH_3 concentration.

Kinetic analysis

The behaviour of ammonia both as a substrate for oxidation and as an inhibitor of methane oxidation was investigated in terms of Michaelis-Menten kinetics. Harrison (1973) and Ferenci *et al.* (1975) reported some kinetic data for methane oxidation by methane-oxidizing bacteria using oxygen uptake as a measure of the initial rates. The work by Suzuki *et al.* (1974) on the kinetics of ammonia oxidation by *Nitrosomonas* also used oxygen uptake as a measure of the initial rates. With *M. trichosporium*, oxygen uptake provided a satisfactory measure of methane oxidation in the presence and absence of ammonia. However, the rates of oxygen uptake due to ammonia oxidation were too low (compared with endogenous rates) to obtain accurate data. The most satisfactory method for measuring rates of ammonia oxidation was by nitrite production.

Rates of methane oxidation, in the presence and absence of ammonia, were determined by following methane disappearance by gas-liquid chromatography. However, initial rates could not be obtained as accurately as with oxygen uptake, and methane concentrations in solution could only be calculated from concentrations in the gas phase. Despite these reservations, kinetic data obtained by both methods were in general agreement.

Ammonia as substrate. Using nitrite production as a measure of the rate of ammonia oxidation, good agreement with Michaelis-Menten kinetics was found. K_m values for ammonia were obtained at different pH values in the presence and absence of formate. The K_m for NH_4^+ at pH 6.5 was 4.1 mM, whereas at pH 7.5 it was 0.6 mM. Recalculating K_m in terms of NH_3 concentration gives essentially similar values of 7.4 and $10.4 \mu\text{M}$ respectively. Repeating the determinations in the presence of formate gave a K_m value for NH_4^+ of 7.1 mM at pH 6.5 and 0.4 mM at pH 7.5, resolving to values of 12.8 and $7.2 \mu\text{M-NH}_3$ respectively. The NH_3 concentrations were calculated from the Henderson-Hasselbalch relationship assuming a pK value of 9.25 for the ionization $\text{NH}_3 + \text{H}_2\text{O} \rightleftharpoons \text{NH}_4^+ + \text{OH}^-$.

Ammonia as an inhibitor of methane oxidation. Using oxygen uptake to measure the initial rate of methane oxidation, good agreement was again obtained with Michaelis-Menten kinetics, ammonia showing competitive inhibition of methane oxidation. At pH values between 6.0 and 8.0 the K_m for methane was 45 to $48 \mu\text{M}$, whereas the K_i for NH_4^+

Fig. 1. The effect of methane on nitrite production from ammonia. Incubations were carried out in 5 ml 20 mM-phosphate buffer pH 6.8 containing 47 mg dry wt bacteria in 30 ml round-bottomed flasks. Ammonia was at a final concentration of 10 mM and methane was added to the gas phase to give a concentration of (●) 8 μ mol and (□) 80 μ mol. ○, No methane added.

decreased from 17.5 mM at pH 6.0 to 0.2 mM at pH 8.0. However, when recalculated in terms of NH_3 concentration the K_i values resolved to 9.8 and 11.2 μ M respectively.

Effect of methane on ammonia oxidation

The effect of methane on ammonia oxidation is complex, both stimulation and inhibition apparently being involved. Using nitrite production to measure the initial rates of ammonia oxidation, the effect of adding different volumes of methane to flasks is shown in Fig. 1. Low levels of methane stimulated nitrite production, whereas high levels were inhibitory. The main effect was to halt nitrite production, indicating more complex interactions between ammonia and methane than are apparent in the initial reaction.

*Interactions of ammonia with *Methylobacterium albus* BG8*

Limited studies were carried out with the type I methane-oxidizing bacterium *Methylobacterium albus* BG8. Significant physiological differences have been observed between type I organisms and type II of which *M. trichosporium* is a representative (Whittenbury *et al.*, 1970; Quayle, 1972; Whittenbury *et al.*, 1975). *Methylobacterium albus* behaved in an essentially similar manner to *M. trichosporium* in its interactions with ammonia, although detailed kinetic studies were not done. However, some differences were noted. Under similar conditions the degree of inhibition of methane oxidation was considerably less in the type I strain, as measured either by oxygen uptake or methane disappearance. In addition, rates of ammonia oxidation were considerably lower. Typical rates for nitrite production were about 0.5 and 4.0 nmol min^{-1} ($\text{mg dry wt bacteria}$) $^{-1}$ for *Methylobacterium albus* and *M. trichosporium* respectively.

DISCUSSION

Our evidence suggests that NH_3 rather than NH_4^+ is involved in interactions with methylobacteria. The situation is thus similar to that in *Nitrosomonas* (Suzuki *et al.*, 1974). In *M. trichosporium* the K_m value for NH_3 as a substrate is the same as the K_i value when NH_3 is acting as an inhibitor of methane oxidation, i.e. about $10\ \mu\text{M}$. This, together with the competitive nature of the inhibition, supports the argument that NH_3 and methane are capable of binding to the same point of attachment in the methane oxygenase system.

The stimulation of ammonia-oxidizing activity by formate together with the known association of formate dehydrogenase with NAD^+ in methylobacteria (Harrington & Kallio, 1960; Johnson & Quayle, 1964) suggests that the reaction involves a mixed function oxidase as shown below:

NADH acts as an electron donor in a similar reaction for methane oxidation in cell-free systems (Ribbons & Michalover, 1970; Ferenci, 1974) but other electron donors are probably involved (Tonge *et al.*, 1975). 3-Hydroxybutyrate also stimulates ammonia-oxidizing activity in *M. trichosporium* and the oxidation of this compound is linked to NAD (Thomson, O'Neill & Wilkinson, 1976).

It has been suggested only relatively recently that the oxidation of ammonia by *Nitrosomonas* involves a mixed function oxygenase (Suzuki & Kwok, 1969). Suzuki (1974) has suggested that the terminal oxidation of nitroxyl (NOH) provides the reducing equivalents for the oxygenase reaction and that the intermediate oxidation of NH_2OH is the only energy-yielding step. The stimulation of the rate, and in some circumstances the extent, of ammonia oxidation by formate and other compounds might therefore suggest that a supply of endogenous reductant is utilized which may be replenished endogenously. However, the ratio of $\text{O}_2:\text{NH}_3$ in this case would be 2:1 and not the observed 1.5:1, which suggests that reducing equivalents are being regenerated during ammonia oxidation.

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