O_2 and H_2O are each the source of one O in NO_2^- produced from NH_3 by *Nitrosomonas*: ^{15}N-NMR evidence

Kristoffer K. Andersson and Alan B. Hooper

Department of Genetics and Cell Biology, University of Minnesota, St. Paul, MN 55108, USA

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The exchange of ^{18}O between $H_2{}^{18}O$ and exogenously added $^{15}N^{16}O_2^-$ which occurs during oxidation of ammonia by *Nitrosomonas* is shown to occur one oxygen at a time. Conditions in which the exchange is diminished (notably the presence of $^{14}NO_2^-$ and CCCP) allowed demonstration that water and dioxygen are each the source of one oxygen in nitrite produced from $^{15}NH_3$. The nitrite produced in the presence of $^{18}O_2$ consisted of 67 and 0% $^{15}N^{18}O^{16}O^-$ and $^{15}N^{18}O^{18}O^-$, respectively. Analysis was made using the ^{18}O-isotope shift in ^{15}N-NMR.

Nitrosomonas *^{15}N-NMR* *Nitrite* *Ammonia* *^{18}O Isotope shift* *Nitrification*

1. INTRODUCTION

The aerobic bacterium *Nitrosomonas* derives energy from the oxidation of ammonia to NO_2^-. Whether oxygen atoms of NO_2^- are from dioxygen or water has not been demonstrated. Authors in [1] achieved incorporation by cells of 7% of one nitrite-oxygen from $^{18}O_2$ as analyzed by mass spectrometry. In [2] and [3] it was demonstrated that cellular oxidation of ammonia to hydroxylamine, a probable intermediate in NO_2^- production, involves incorporation of oxygen from dioxygen. Thus, assuming that hydroxylamine is an intermediate and that the oxygen of hydroxylamine is not lost by hydrolysis, at least one of the two oxygens of NO_2^- is from dioxygen. In [4], utilizing analysis by $^{15}N(^{18}O)$-NMR, it was demonstrated that at least 20% of one of the oxygens of NO_2^- produced from ammonia by intact cells originated from dioxygen. A rapid cell-catalyzed exchange of oxygen between NO_2^- and water made identification of the source of the second nitrite-oxygen impossible. The oxygen exchange reaction required concomitant oxidation of ammonia or hydroxylamine by cells. Here, means have been found to partially inhibit the exchange of oxygens in $^{15}NO_2^-$ produced from $^{15}NH_3$ by *Nitrosomonas*. During cellular oxidation of ammonia to NO_2^-, one oxygen is shown to originate from dioxygen and the other from water.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PMS, *N*-methylphenazonium methosulfate

2. MATERIALS AND METHODS

2.1. Nitrosomonas europaea

Growth of *Nitrosomonas europaea*, incubation procedures and ^{15}N-NMR analysis was carried out as in [4]. Washed cells were suspended to 800 mg wet wt/ml in 0.5 M potassium phosphate solution, pH 8.0. Storage at 4°C was no longer than 2 days. Incubation was initiated by adding the suspension of bacteria with a Hamilton syringe through a serum stopper into a 4-ml reaction mixture in a 15-ml glass centrifuge tube. The reaction solution (0.2 M potassium phosphate, pH 8, in $H_2{}^{16}O$ or $H_2{}^{18}O$, ^{14}N- or ^{15}N-compounds and equilibrated with pure $^{18}O_2$ or $^{16}O_2$, as indicated) contained 10 mg wet wt/ml cells unless otherwise indicated. After removal of cells by sedimentation, NMR spectra of the 4 ml supernatant were measured with a Nicolet NJ-300 spectrometer at 30.42 MHz

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(7.05 T) at 25°C for the indicated number of scans. The absorption of $^{15}N^{16}O_2^-$ in neutral media was defined as 0 ppm. In ^{15}N-NMR the position of the $^{15}NO_2^-$ resonance is shifted downfield when a ^{16}O is replaced by an ^{18}O [4,5]. The peak heights are directly proportional to the relative concentration of the 3 NO_2^- species ($^{15}N^{16}O^{16}O^-$, $^{15}N^{16}O^{18}O^-$ and $^{15}N^{18}O^{18}O^-$). $^{14}NO_2^-$ is not detected. A 1 Hz line-broadening factor was employed. At pH 7.5 chemical exchange of oxygen between water and NO_2^- is less than 3% in 24 h.

2.2. *Materials*

$^{15}NH_3$ sulfate and sodium $^{15}NO_2$ (99% enriched) were from Stohler Isotopes (Waltham, MA). $^{18}O_2$ (g) and $H_2{}^{18}O$ (at least 98% enriched) were from Monsanto Research Corp./US Department of Energy (Miamisburg, OH). CCCP, PMS and hydrazine were from Sigma Chemicals (St. Louis, MO). Nitrapyrin was a gift of Dow Chemicals (Midland, MI). All other chemicals were analytical grade. Glass distilled water was used.

2.3. *Quantitative analysis of the exchange of oxygen between water and NO_2^-*

Following oxidation of $^{15}NH_3$ by cells in presence of $^{18}O_2$ and $H_2{}^{16}O$, any ^{18}O-containing $^{15}NO_2^-$ existed in the presence of a great excess of $H_2{}^{16}O$ so that exchange of oxygen between $^{15}N^{18}O_2^-$ and $H_2{}^{16}O$ could be considered essentially irreversible. Cell-catalyzed exchange of nitrite-oxygen occurred one oxygen at a time (see results) as is the case for the purely chemical exchange [5]. As developed in [5], for a given rate constant (k) the loss from NO_2^- of an ^{18}O atom by the exchange reaction is expressed by:

$$N^{18}O^{18}O^- \xrightarrow[k]{H_2{}^{16}O} N^{18}O^{16}O^- \xrightarrow[1/2\,k]{H_2{}^{16}O} N^{16}O^{16}O^-$$

The resulting set of linear first order differential equations were derived and solved by the method of variation of parameter [5]. A solution to the differential equations (relative amounts of $^{15}N^{18}O^{18}O^-$, $^{15}N^{18}O^{16}O^-$ and $^{15}N^{16}O^{16}O^-$ as a function of time) was plotted for an arbitrarily chosen value of k to give the relative amounts of the 3 isotopic combinations which could be present at any given stage in the reaction (regardless of the true value of k). Given a postulated starting species (i.e., $^{15}N^{18}O^{18}O^-$ here) the plot allows one to predict the size of one peak from the measured ratio of the other two peaks.

3. RESULTS AND DISCUSSION

3.1. *Cell-catalyzed nitrite-water exchange of oxygen occurs one oxygen at a time in exogenously added nitrite*

At equilibrium, an exchange of oxygen atoms $^{15}N^{16}O^{16}O^-$ with $H_2{}^{16}O$ and $H_2{}^{18}O$ (0.4:0.6) results in $^{15}N^{16}O^{16}O^-$, $^{15}N^{16}O^{18}O^-$ and $^{15}N^{18}O^{18}O^-$ in a ratio of $(0.4)^2:(0.4 \times 0.6 \times 2):(0.6)^2$ or 0.16:0.48:0.36. If the exchange had occurred by the simultaneous removal of the 2 nitrite oxygens and subsequent addition of 2 water oxygens ($NO_2^- \longrightarrow$ 'N$^-$': 'N$^-$' + 2O $\longrightarrow NO_2^-$) the probability of adding ^{16}O or ^{18}O would be based simply on the $H_2{}^{16}O:H_2{}^{18}O$ ratio so that the ratio of $^{15}N^{16}O^{18}O^-:{}^{15}N^{18}O^{18}O^-$ would be 0.48:0.36 (the same as the equilibrium value) at all times during the approach to equilibrium. The two experiments shown in fig.1 indicate that nitrite-oxygens were exchanged one oxygen at a time. Fig.1A shows the isotopic composition of 10 mM $^{15}O_2^-$ which had been incubated for 30 min in the presence of cells, 60% $H_2{}^{18}O$ and 1 mM $^{14}NH_3$; this yielded 1 mM $^{14}NO_2^-$. Fig.1B shows 20 mM

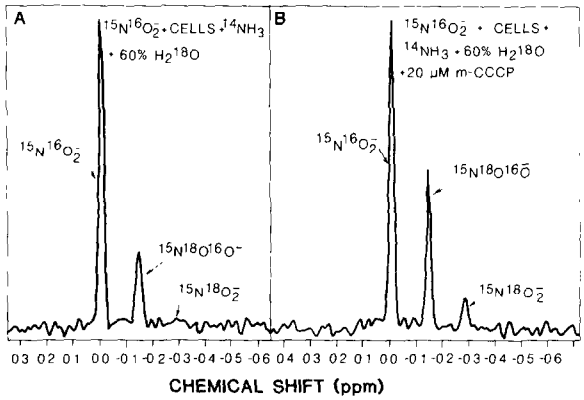

Fig.1. Exchange of oxygen between $^{15}N^{16}O^{16}O^-$ and water during oxidation of $^{14}NH_3$ by *Nitrosomonas*. (A) Incubation of cells in 60% $H_2{}^{18}O$, 1 mM $^{14}NH_3$ and 10 mM $^{15}NO_2^-$ was for 30 min; 1 mM $^{14}NO_2^-$ was produced. The NMR spectrum is the result of 10820 scans. (B) Incubation of cells in 60% $H_2{}^{18}O$, 50 mM $^{14}NH_3$, 20 mM $^{15}NO_2^-$ and 20 μM CCCP for 2 h; 4.3 mM $^{14}NO_2^-$ was produced. 2300 scans.

$^{15}NO_2^-$ which had been incubated for 2 h in 60% $H_2{}^{18}O$, 20 μM CCCP (an inhibitor of ammonia oxidation) and 50 mM $^{14}NH_3$; in this experiment cells produced 4.3 mM $^{14}NO_2^-$. The relative amounts of $^{15}N^{16}O^{16}O^-$:$^{15}N^{16}O^{18}O^-$:$^{15}N^{18}O^{18}O^-$ were 0.8:0.2:0.0 and 0.62:0.32:0.06 for fig.1A and B, respectively. In neither case has the exchange reaction reached equilibrium (where the ratio would have been 0.16:0.48:0.36). Further, the ratios of $^{15}N^{16}O^{18}O^-$:$^{15}N^{18}O^{18}O^-$ in either case were significantly different from 0.48:36, the value expected if 2 oxygens were exchanged simultaneously. The results indicate that under the conditions of fig.1 (in presence or absence of CCCP), exchange of oxygen between exogenously added NO_2^- and water as catalyzed by *Nitrosomonas* took place one oxygen at a time as is seen with the acid-catalyzed chemical exchange reaction [5].

3.2. *Partial inhibition of exchange of oxygens of $^{15}NO_2^-$ produced from $^{15}NH_3$*

Interference with the exchange of oxygen between water and $^{15}NO_2^-$ produced from $^{15}NH_3$ could be achieved by:

(i) addition of exogenous $^{14}NO_2^-$ [4];
(ii) incubation with $^{15}NH_3$ for short times so as to minimize the time of contact of $^{15}NO_2^-$ with cells;
(iii) incubation with relatively low concentrations of cells (thus giving low rates of ammonia oxidation); and
(iv) the presence of low concentrations of hydrazine (5 mM), PMS (5–20 μM) nitrapyrin (5–60 μM) or CCCP (5–20 μM).

The extent of interference with the exchange generally increased with concentration of added compound but in a complex way. The effect of CCCP on the exchange is reflected in fig.1B; in the corresponding control lacking CCCP the exchange was complete [4]. The greatest diminution of the exchange has been observed with CCCP. Possible mechanisms for the exchange reaction include:

(i) acid-catalyzed exchange in the periplasmic space or membrane lumen of *Nitrosomonas* where a low pH may be associated with an energy-linked proton gradient;
(ii) reversible hydrolysis of NO_2^- as proposed [6] for NO_2^- reductase of denitrifying bacteria. The latter may, in fact, occur as the final stage of NO_2^- synthesis in *Nitrosomonas*. The effects of decreasing the flux of ammonia oxidation or the presence of CCCP, hydrazine, PMS or nitrapyrin can be rationalized in terms of either hypothesis. We note that CCCP, hydrazine, PMS and nitrapyrin are inhibitors of ammonia oxidation in *Nitrosomonas* [7] CCCP is an uncoupler [8]; and PMS and CCCP [9] may function as redox mediators.

3.3. *Nitrite produced by oxidation of ammonia contains one oxygen from dioxygen and one from water*

We have not been able to completely inhibit the exchange reaction. Our approach is therefore to retard the exchange reaction as much as possible and ask if the ratio of $^{15}N^{16}O^{16}O^-$:$^{15}N^{16}O^{18}O^-$:$^{15}N^{18}O^{18}O^-$ is consistent with either $^{15}N^{18}O^{18}O^-$ or $^{15}N^{18}O^{16}O^-$ having been the initial product of oxidation of $^{15}NH_3$ in presence of $^{18}O_2$ and $H_2{}^{16}O$. The effect of the exchange of oxygen of $^{15}NO_2^-$ with $H_2{}^{16}O$ in the two cases can be represented as:

(i) $^{15}N^{18}O^{16}O^- \longrightarrow {}^{15}N^{16}O^{16}O^-$; or
(ii) $^{15}N^{18}O^{18}O^- \longrightarrow {}^{15}N^{16}O^{18}O^- \longrightarrow {}^{15}N^{16}O^{16}O^-$.

If one assumes that the exchange occurred one oxygen at a time (fig.1) and a pseudo first order reaction mechanism operates the exchange, then the two cases may be analyzed as in [5]. In (i) the exchange involves simply the first order disappearance of $^{15}N^{18}O^{16}O^-$ so that a $^{15}N^{18}O^{18}O^-$ peak should not occur at any time. In (ii) the size of the expected $^{15}N^{18}O^{18}O^-$ peak can be predicted from the ratio of the $^{15}N^{16}O^{16}O^-$ and $^{15}N^{16}O^{18}O^-$ peaks (see section 2.3). One assumes that at the beginning of the exchange reaction $^{15}N^{16}O^{16}O^-$ was not present, whereas, at equilibrium, neither $^{15}N^{16}O^{18}O^-$ nor $^{15}N^{18}O^{18}O^-$ will be present. During the course of equilibration all 3 species should appear.

$^{15}NO_2^-$ was produced from $^{15}NH_3$ under conditions in which the exchange reaction was partially inhibited. Decreasing the concentration of cells to 10 mg/ml (rather than 40 mg/ml as in [4]) allowed the use of only 25 mM (rather than 100 mM) extracellular $^{14}NO_2^-$ to obtain an incorporation of 24%, of one ^{18}O from dioxygen into $^{15}NH_3$-derived nitrite. The presence of 60 μM nitrapyrin allowed incorporation of 30% of one ^{18}O into NH_3-derived nitrite. Given our signal-to-noise ratio the resulting ratios 76:24:0 or 70:30:0, respectively, could have been obtained by exchange starting with either $^{15}N^{16}O^{18}O^-$ or

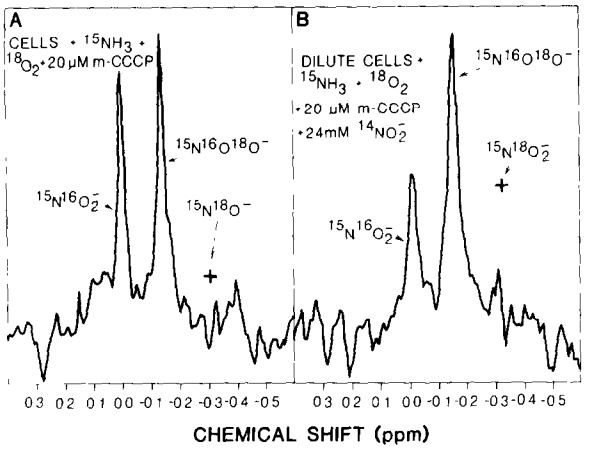

Fig.2. Oxygen isotope composition of $^{15}NO_2^-$ formed by oxidation of $^{15}NH_3$ by *Nitrosomonas* in $^{18}O_2$. (A) Incubation of 10 mg cells/ml, 50 mM $^{15}NH_3$, 20 μM CCCP was for 2 h in an atmosphere of $^{18}O_2$; 3 mM $^{15}NO_2^-$ was produced. The NMR spectrum is the result of 5778 scans. (B) Incubation of 2 mg/ml cells, 50 mM $^{15}NH_3$, 24 mM $^{14}NO_2^-$, 20 μM CCCP for 14 h; 3.4 mM $^{15}NO_2^-$ was produced, 8688 scans. The points (+) indicate the minimum amount of $^{15}N^{18}O^{18}O^-$ expected to remain had it been the initial product of oxidation of $^{15}NH_3$.

$^{15}N^{18}O^{18}O^-$. The results confirmed that dioxygen was the source of one oxygen but were not informative as to the source of the second oxygen. Significant production of $^{15}N^{18}O^{16}O^-$ also occurred with added hydrazine or PMS.

More instructive results were obtained in presence of CCCP. A result typical of 3 experiments is shown in fig.2A; 3 mM of $^{15}NO_2^-$ was produced after a 2-h incubation of 50 mM $^{15}NH_3$, 10 mg cells/ml, 20 μM CCCP and $^{18}O_2$ (g) in $H_2{}^{16}O$. Production of $^{15}N^{18}O^{18}O^-$ can be ruled out by the data. Based on the ratio of the $^{15}N^{16}O^{16}O^-$:$^{15}N^{16}O^{18}O^-$ peaks (0.86) the corresponding $^{15}N^{18}O^{18}O^-$ peak should have been at least 1/3 of the $^{15}N^{16}O^{16}O^-$ peak (13% of total NO_2^-, indicated by + in fig.2A) if $^{15}N^{18}O^{18}O^-$ had been the initial product of oxidation of $^{15}NH_3$. In fact $^{15}N^{18}O^{18}O^-$ was not observed. We note that, since NO_2^- was produced continuously, the true time of incubation with cells of the $^{15}N^{18}O^{18}O^-$ produced late in the incubation would have been less than $^{15}NO_2^-$ produced early in the incubation. Thus the theoretical $^{15}N^{18}O^{18}O^-$ peak height (13%) is a minimum value.

In the experiment shown in fig.2B, exchange of oxygen between $^{15}NO_2^-$ and $H_2{}^{16}O$ was limited by competition with 24 mM $^{14}NO_2^-$, inclusion of 20 μM CCCP and incubation with a dilute suspension (2 mg/ml) of cells. From the ratio of $^{15}N^{16}O^{16}O^-$:$^{15}N^{16}O^{18}O^-$ (0.5) the $^{15}N^{18}O^{18}O^-$ peak should have been at least the same size as $^{15}N^{16}O^{16}O^-$ (25% of total $^{15}NO_2^-$ indicated by + in fig.2B) if $^{15}N^{18}O^{18}O^-$ had been the initial product of oxidation of $^{15}NH_3$. In fact, $^{15}N^{18}O^{18}O^-$ was not observed.

The present results indicate that dioxygen and water each donate one atom of oxygen to nitrite produced from ammonia by *Nitrosomonas*. We note that the conclusion requires that the O-exchange in NO_2^- produced endogenously by cells occurs one oxygen at a time as it does in exogenously added NO_2^- (fig.1). Although we cannot exclude a difference in mechanism, the stimulation of production of $^{15}N^{18}O^{16}O^-$ in the presence of exogenously added $^{14}NO_2^-$ suggests that exogenous NO_2^- can compete in the exchange reaction with endogenously produced NO_2^- and thus that the mechanisms are the same.

Based on the results in [2] and [3] an atom of oxygen from dioxygen is added to ammonia to form hydroxylamine. We note that oxygenation of ammonia may involve production of water (i.e., typical of a monooxygenase) or the incorporation of both atoms of dioxygen into an N-substrate (i.e., typical of a dioxygenase). Assuming that hydroxylamine is, indeed, an intermediate our results indicate that the second oxygen in biological nitrite synthesis comes from water. Thus nitrite is apparently produced by a water-utilizing hydroxylamine dehydrogenase; $NH_2OH \longrightarrow 3H^+ + 4e^- + NO^+$; $NO^+ + H_2O \longrightarrow HONO + H^+$. We speculate that the second part of the reaction may account for the nitrite–water exchange reaction.

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