Definitive ^{15}N NMR evidence that water serves as a source of 'O' during nitrite oxidation by *Nitrobacter agilis*

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Received 13 December 1982

Using ^{18}O isotope shifts in ^{15}N NMR it has been shown that during oxidation of nitrite to nitrate by *Nitrobacter agilis*, the third 'O' in nitrate originates from water.

Nitrobacter agilis *Nitrite oxidation* ^{15}N *NMR* ^{15}N–^{18}O *isotope shift*

1. INTRODUCTION

The chemolithotropic nitrifying bacterium, *Nitrobacter agilis* oxidises nitrite to nitrate thus generating ATP and reducing equivalents (NADH) for growth. It has been shown that the source of oxygen during nitrite oxidation by *N. agilis* is water [1] based on the incorporation of 0.044–0.078 atom % ^{18}O into nitrate from 82 atom % $H_2{}^{18}O$. Because we have been unable to demonstrate respiration-driven proton ejection in oxygen pulse experiments [2] we considered the possibility that, should the proton pump mechanism be absent, the bacterium might synthesize ATP by substrate type oxidative phosphorylation including a mixed anhydride between either NO_3^- and PO_4^{2-} or NO_3^- and ADP. Should this concept be correct, the 'O' in NO_3^- produced by NO_2^- oxidation in *N. agilis* would come from PO_4^{2-}. The ^{18}O isotope shift in ^{15}N NMR has recently been used [3] to demonstrate H_2O–NO_2^- exchange reactions in *Nitrosomonas europaea*. The main advantage of this technique is that the reactants and products can be studied directly. Here, we report on the incorporation of ^{18}O from $H_2{}^{18}O$, $^{18}O_2$ and $P^{18}O_4^{2-}$ during the oxidation of NO_2^- to NO_3^- by washed cells of *N. agilis*, using the secondary isotope effect; i.e., the shift in the ^{15}N resonance of NO_3^- when ^{16}O is substituted for ^{18}O [4].

2. MATERIALS AND METHODS

2.1. *Bacterium and growth conditions*

Nitrobacter agilis ATCC 14123 was grown in 8-l batches for 5 days with vigorous aeration in an inorganic medium as in [5]. The cells harvested by continuous flow centrifugation at 4°C as in [6] were washed several times with cold 100 mM sodium phosphate, 5 mM K_2CO_3 buffer (pH 7.8) and finally suspended in the same buffer at about 500–600 mg wet wt/ml.

2.2. *Isotope experiments*

All the experiments were carried out in 50-ml Erlenmeyer flasks at 28°C in a waterbath shaker (120 rev./min). Freshly harvested cells oxidised about 50 nmol of $NO_2^-.min^{-1}.mg\ wet\ wt^{-1}$. In thick cell suspensions, cells tend to become anaerobic quickly so that NO_2^- oxidation slows down. Thus oxygen was generated by the addition of catalase-H_2O_2 [6]. The following experiments were done:

(i) 1 ml cell suspension (~500 mg wet wt) was diluted to 10 ml in 100 mM phosphate, 5 mM carbonate buffer;

Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

(ii) 1 ml cell suspension was diluted to 10 ml in the same buffer, and the flask closed with a serum septum. The flask was evacuated with an Edwards 2-stage pump and filled with 100% $^{18}O_2$ (99.2 atom % ^{18}O);
(iii) 1 ml cell suspension was added with 1 ml each of 200 mM phosphate, 10 mM carbonate (pH 7.8) and 97 atom % $H_2{}^{18}O$;
(iv) 1 ml cell suspension was centrifuged in an Eppendorf tube at 13000 × *g* for 5 min and the pellet resuspended in 10 ml of ^{18}O phosphate-5 mM carbonate buffer (pH 7.8).

To all the cell suspensions in 50 ml Erlenmeyer flasks, was added catalase (1 mg) and 40% v/v H_2O_2 (5 μl) (except for expt b), followed by incubation at 28°C in water bath shaker. Then 50 μmol $K^{15}NO_2$ (97 atom % ^{15}N) was added to each flask to start the reaction. Aliquots, 5–10 μl, were withdrawn from the reaction mixtures to check NO_2^- concentration as in [7]. As soon as the nitrite was utilized completely, another 50 μmol of $^{15}NO_2^-$ was added and the reaction continued until at least 200 μmol of total nitrite had been oxidised to nitrate. The initial rate of NO_2^- oxidation was relatively fast (50–70 μmol.mg wet $wt^{-1}.min^{-1}$) but after 2–3 additions of NO_2^- it slowed down presumably because of NO_3^- accumulation. This effect was more pronounced when the total reaction volume was 3 ml (expt c). Cells in 10 ml (expts a,b and d) oxidised about 400 μmol of NO_2^- in 4–5 h, whereas in a 3 ml volume (expt c) they required 7–8 h. At the end of the reaction, cell suspensions were centrifuged at 20000 × *g* for 10 min at 4°C and the supernatant fractions were carefully dispensed with a Pasteur pipette. The volume of each fraction was made 10 ml with phosphate-carbonate buffer, the pH adjusted to 8.0 if needed and then immediately frozen in liquid N_2 until used in NMR studies.

2.3. *^{15}N NMR analysis*

30.42 MHz ^{15}N-NMR spectra were obtained on a Bruker CXP 300 NMR spectrometer operating at a field strength of 7.05 T. Spectra were acquired from 2 dm^{-3} samples in 10 mm NMR tubes as the result of about 200 scans into an 8 K data table. A 15°C (10 μs) pulse was used with a 4.1-s recycle time and no ^1H-decoupling. After acquisition, a line broadening of 0.1 Hz was applied, together with apodisation. The data were zero filled to 16 K before Fourier transformation.

2.4. *Isotopes*

^{15}N-labelled HNO_3 (97 atom % ^{15}N) was purchased from Isomet, NJ; $K^{15}NO_3$ was prepared by the titration of $H^{15}NO_3$ with KOH; $K^{15}NO_2$ was prepared by the reduction of $K^{15}NO_3$ in the presence of lead at 420°C; $H_2{}^{18}O$ (97 atom % ^{18}O) was obtained from Merck Sharp and Dohme (Montreal); $^{15}N,{}^{18}O$-labelled nitrate standards were prepared by the method in [8]; $H_3P^{18}O_4$ (~97 atom % ^{18}O) was prepared by the reaction of $H_2{}^{18}O$ on PCl_5.

All other chemicals used in the study were the highest purity grade available. Double glass distilled water was used throughout.

3. RESULTS

The signals of various $^{15}N^{18}O$ nitrate standards (fig.1A) were essentially as in [3], but only 3 peaks were observed, corresponding to $^{15}N^{16}O_3^-$, $^{15}N^{16}O_2{}^{18}O^-$ and $^{15}N^{16}O^{18}O_2^-$ as confirmed by spiking the $^{15}N^{16}O_3^-$ resonance. The peaks were well resolved and separated by 1.71 Hz (0.0563 ppm). A visible signal was observed after a few scans when the concentration of $^{15}NO_3^-$ was more than 40 mM. Smaller concentrations required longer accumulation time.

When the cells were incubated with $^{15}NO_2^-$ (expt a) with $H_2{}^{16}O$ in 100 mM $P^{16}O_4^{2-}$ buffer, only one resonance was observed which corresponded to $^{15}N^{16}O_3^-$ (not shown). In fig.1B, the NMR spectrum of the product of $^{15}NO_2^-$ oxidation in the presence of 100% $^{18}O_2$ (expt b) is shown. Again only one peak was observed with an isotopic configuration of $^{15}N^{16}O_3^-$ indicating that none of the 'O' in nitrate produced by $^{15}NO_2^-$ oxidation was derived from $^{18}O_2$. When cells were incubated with $^{15}NO_2^-$ and $H_2{}^{18}O$ (expt c) two major peaks and a minor one were observed, separated by 1.71 Hz and representing $^{15}N^{16}O_3^-$, $^{15}N^{16}O_2{}^{18}O^-$ and $^{15}N^{16}O^{18}O_2^-$, respectively (fig.1C). The ratio of the areas of 3 peaks was 31.6:6:1. Thus $^{15}N^{16}O_2{}^{18}O^-$ and $^{15}N^{16}O^{18}O_2^-$ isotope combinations were about 19% and 3.2%, respectively, of $^{15}N^{16}O_3^-$. Expt d was designed to check for substrate level phosphorylation in *Nitrobacter* that might involve an anhydride-like intermediate between either NO_3^- and PO_4^{2-} or ADP and NO_3^-. Thus cells were incubated in ^{18}O phosphate (all 4 'O' atoms labelled with ^{18}O). Fig.1D shows the spectrum of nitrate

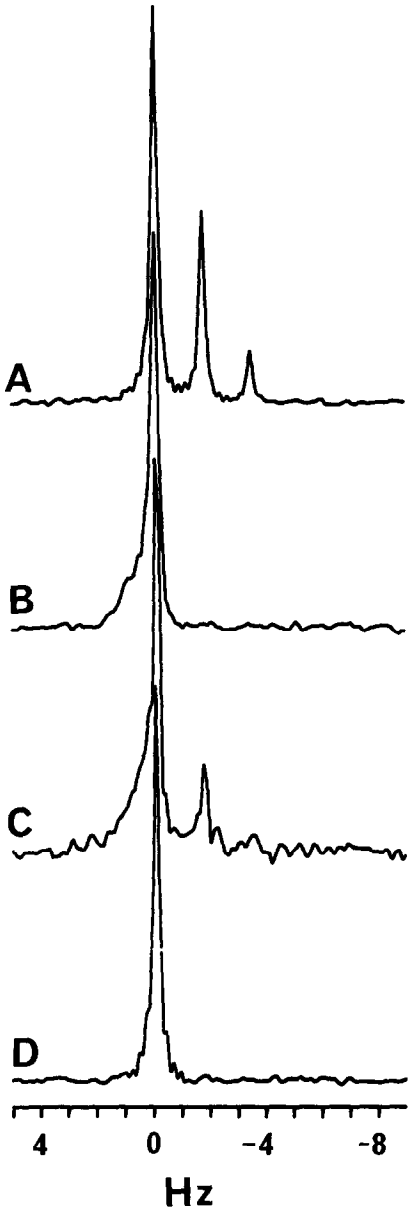


Fig.1. NMR of $^{18}O/^{16}O$ derivatives of nitrate: (A) 100 mM standard $^{15}N^{18}O$ nitrate derivatives produced by chemical exchange; (B) 40 mM, $^{15}N^{16}O_3^-$ produced by cells in presence of $^{18}O_2$; (C) 20 mM ($^{15}N^{16}O_3^-$ + $^{15}N^{16}O_2{}^{18}O^-$ + $^{15}N^{16}O^{18}O_2^-$) produced by cells in presence of $H_2{}^{18}O$; (D) 50 mM $^{15}N^{16}O_3^-$ produced by cells in presence of $P^{18}O_4^{2-}$. For further details see section 2.

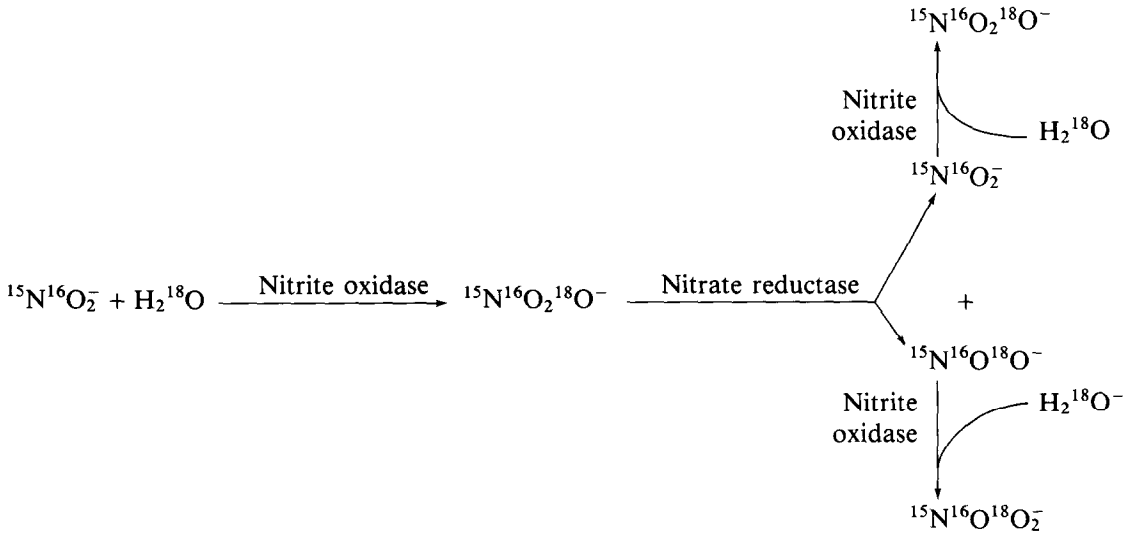
produced from the oxidation of $^{15}NO_2^-$ in the presence of ^{18}O phosphate (100 mM). Only one peak was observed which corresponded to $^{15}N^{16}O_3^-$ indicating that none of the 'O' in $^{15}NO_3^-$ is derived from $P^{18}O_4^{2-}$ during nitrite oxidation by *N. agilis*. In another experiment when cells were incubated for 18 h with $P^{18}O_4^{2-}$ and $^{15}NO_2^-$, the NMR spectrum was similar to that observed in fig.1D (expt c). Thus there appears to be no measurable biological or chemical exchange of ^{18}O between either $P^{18}O_4^{2-}$ and H_2O, $P^{18}O_4^{2-}$ and $^{15}NO_3^-$ or $P^{18}O_4^{2-}$ and $^{15}NO_2^-$.

4. DISCUSSION

One of the advantages of using ^{15}N NMR is that with the aid of stable isotopes (^{15}N and ^{18}O) the reactants and products of a biochemical reaction can be analysed directly. This overcomes any dilution or exchange reactions associated with the processing of the samples. The technique of ^{18}O isotope shift in ^{15}N NMR has recently been used in [3] to demonstrate the 'O' exchange reactions between NO_2^- and H_2O catalysed by *Nitrosomonas europaea*. This is a powerful technique to study the oxidations and reductions of inorganic nitrogen compounds; e.g., NH_4^+, NH_2OH, NO_2^- and NO_3^-. Here, we show that during the oxidation of nitrite by *Nitrobacter agilis* the 3rd 'O' in NO_3^- arises from water. Our results substantiate the mass spectrometric data in [1].

As shown in fig.1C for cells incubated with $^{15}NO_2^-$ and $H_2{}^{18}O$ (expt c) 3 resonances in the NMR-spectrum represented $^{15}N^{16}O_3^-$ (100%), $^{15}N^{16}O_2{}^{18}O^-$ (19%) and $^{15}N^{16}O^{18}O_2^-$ (2.3%). As the incubation mixture contained 1 ml of 97 atom % $H_2{}^{18}O$ in a final volume of 3 ml, the final enrichment of ^{18}O would be about 32%. The observed enrichment of ^{18}O in $N^{18}O_3^-$ produced by $^{15}NO_2^-$ oxidation was 19 + 3.2 = 22.2%. If all the 'O' in NO_3^- is derived from water, the $^{15}N^{16}O_2{}^{18}O^-$ peak should be about 32% of $^{15}N^{16}NO_3^-$. The $^{15}NO_2^-$ used in these experiments prepared by the reduction of $^{15}NO_3^-$ with Pb was found to contain about 30% $^{15}NO_3^-$ (analysed by ^{15}N NMR). When this correction is applied, the ratio of $^{15}N^{18}O_3^-$ and $^{15}N^{16}O_3$ produced by the oxidation of $^{15}NO_2^-$ by *N. agilis* would be close to the theoretically expected value (32%). This proves that all the ^{18}O in $^{15}N^{18}O_3^-$ was derived from $H_2{}^{18}O$. The oxidation of nitrite to nitrate by *N. agilis* requires only one oxygen atom which is supplied by water [1,9]. The

appearance of $^{15}N^{16}O^{18}O_2^-$ resonance in ^{15}N-NMR spectrum is thus unusual. A chemical exchange of ^{18}O between $H_2{}^{18}O$ and $^{15}N^{16}O_2{}^{18}O^-$ at alkaline pH is highly unlikely. Thus a possible explanation of the appearance of $^{15}N^{16}O^{18}O_2^-$ could be associated with the recycling of $^{15}NO_2^-$ by nitrite oxidase and nitrate reductase [9,10] in thick cell suspensions which tend to become anaerobic.



The results of experiments (a) and (d) proved that none of the oxygen in NO_3^- was derived from either $^{18}O_2$ or $P^{18}O_4^{2-}$ because the only peak observed had an isotopic configuration of $^{15}N^{16}O_3^-$. This rules out the possibility of an P-O-N type intermediate during nitrite oxidation by *N. agilis*. The results presented here constitute definitive evidence that during NO_2^- oxidation, H_2O, and not O_2 gas (air), serves as 'O' donor.

ACKNOWLEDGEMENTS

This work was supported by a generous grant to D.J.D.N. from the Australian Research Grants Committee. S.K. is grateful to the University of Adelaide for a postgraduate award. We also thank Dr T.C. Hollocher for helpful discussion.

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