Vol. 91, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 14, 1979 Pages 10-16

NITRIC OXIDE AS AN INTERMEDIATE IN DENITRIFICATION: EVIDENCE FROM NITROGEN-13 ISOTOPE EXCHANGE

Mary K. Firestone[a], Richard B. Firestone[b], and James M. Tiedje[c]

[a]Dept. of Soils and Plant Nutrition, [b]Lawrence Berkeley Laboratory, University of California, Berkeley, CA 94720 and [c]Dept. of Crop and Soil Sciences, Dept. of Microbiology and Public Health, Michigan State University, E. Lansing, MI 48824

Received October 8, 1979

SUMMARY: Label exchange studies were used to investigate the role of nitric oxide as an intermediate of denitrification in Pseudomonas aureofaciens and Pseudomonas chlororaphis. The $[^{13}N]N$ from $[^{13}N]NO_2^-$ readily exchanged with pools of added, nonlabeled NO, with 54% of the $[^{13}N]N$ appearing in a pool of 7.2×10^{-3} atm NO in P. aureofaciens. These results suggest that NO is either an intermediate in the reductive sequence or is in rapid equilibrium with an unidentified intermediate.

INTRODUCTION:

Denitrification, the use of nitrogen oxides as electron acceptors during anaerobic respiration, occurs in a number of genera of bacteria. This process can be of significant agronomic importance because it converts the biologically available ionic forms of N (NO_3^- and NO_2^-) to gaseous products N_2 and N_2O. It has been commonly accepted that denitrification occurs via the following sequence of intermediates (1):

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

Currently, however, there is uncertainty as to the role of NO in the reductive pathway (2,3,4) and this uncertainty has been enhanced by ^{15}N tracer studies in Pseudomonas aeruginosa (2). In this paper we report the results of ^{13}N tracer studies using two denitrifying strains and discuss the compatability of these findings with several proposed pathways.

METHODS:

Pseudomonas aureofaciens (ATCC 13985; assigned to Pseudomonas fluorescens biotype E by Stanier, Palleroni, and Doudoroff [5]) was obtained from the American Type Culture Collection and Pseudomonas chlororaphis (assigned to Pseudomonas fluorescens biotype D [5]; from the strain deposited with ATCC as 17809) was obtained from G. E. Becker, University of Iowa. These organisms

0006-291X/79/210010-07$01.00/0

were chosen for study because of their inability to reduce N_2O to N_2 (unpublished data and personal communications from G. E. Becker). The accumulation of N_2O as the terminal product of denitrification simplified experimental procedure and data interpretation. Cells were grown anaerobically in nutrient broth plus 10 mM NO_3^- and harvested in late exponential or early stationary phase. After washing twice, the cells were resuspended in nutrient broth to about 0.30 mg dry cell mass per mℓ.

Twenty-five milliliters of the cell suspension and 0.25 mℓ of Antifoam A (Sigma Chemical; antifoam diluted 1:500 with water which contained 1 drop of Tween 80 per 25 mℓ as a surfactant) were placed in a 160-mℓ flask with side-port septum for gas or substrate addition. The flask was placed on a magnetic stirrer, attached to a gas recirculating system, and flushed with argon to remove O_2. After the exclusion of O_2 was confirmed, argon was continuously bubbled through the cell suspension (100 mℓ per min) and circulated through a 1 mℓ sampling loop in a Perkin Elmer 910 gas chromatograph (conditions of operation previously described [6]). The gas effluent from the chromatograph column passed either through a ^{63}Ni electron capture detector or through a gas-flow proportional counter for quantitation of $[^{13}N]$ gas (7).

The $[^{13}N]$ substrate was generated at the Michigan State University Cyclotron using previously reported procedures (7). Purified $[^{13}N]NO_2^-$ was prepared by Cd reduction (8) and the purity of the labeled substrate was periodically confirmed to be greater than 99% $[^{13}N]NO_2^-$ by HPLC analysis (7).

Two minutes prior to the addition of labeled substrate, the desired quantity of NO was injected. The time of injection of about 1 mCi of $[^{13}N]NO_2^-$ (activity at time of injection) plus 1.34 μmoles KNO_2 carrier, was recorded as zero-time. Samples of the headspace gas were periodically analyzed for about 1 h after substrate addition. The ^{13}N data from gas analysis were corrected for background, half-life, and solubility.

RESULTS:

Production of $[^{13}N]NO$ and $[^{13}N]N_2O$ from $[^{13}N]NO_2^-$ by P. aureofaciens and P. chlororaphis in the presence and absence of added nonlabeled NO is shown in Table 1. Maximum accumulation of $[^{13}N]NO$ occurred between 25 and 27 min in both strains; with P. aureofaciens about 54% of the ^{13}N appeared in the NO pool (7.2×10^{-3} atm NO added) and with P. chlororaphis about 22% of the label accumulated as $[^{13}N]NO$ in the presence of 1.4×10^{-3} atm NO. The small amount of $[^{13}N]NO$ indicated in the presence of autoclaved P. chlororaphis cells is within the normal counting error of background.

The time course of labeled gas production by P. aureofaciens in the presence of two quantities of added nonlabeled NO is shown in Fig. 1. In the presence of 0.4×10^{-3} atm NO (Fig. 1A), a small percentage of the ^{13}N label accumulated as $[^{13}N]NO$; while in the presence of 7.2×10^{-3} atm NO (Fig. 1B), $[^{13}N]NO$ was the major species present until about 45 min. Total $[^{13}N]$ gas accumulation ceased between 35 to 40 min. Experiments run immediately prior

Table 1. [^{13}N]NO and [^{13}N]N_2O produced by P. aureofaciens and P. chlororaphis in the presence and absence of added NO.

NO added (10^{-3} atm)	NO added (μM)[b]	% of ^{13}N label as [^{13}N]NO	% of ^{13}N label as [^{13}N]N_2O
P. aureofaciens			
0	0	0.0	101.7
0.4	0.7	2.7	76.0
7.2	14.0	54.2	39.4
P. chlororaphis			
0	0	0.8	81.8
0.4	0.7	4.8	80.6
1.4	2.8	21.9	77.7
(Sterile)[c]			
1.4	2.8	0.5	0.0

[a]Headspace gas from P. aureofaciens was sampled at 27 min, P. chlororaphis at 25 min after the addition of 1.34 µmoles KNO_2 plus [^{13}N]NO_2^-. (53.6 µM NO_2^-).

[b]Quantities of NO added are expressed in atm and as the calculated initial concentration of nonlabeled NO in solution (9).

[c]P. chlroraphis sterile control contained cells autoclaved for 20 min immediately prior to experiment.

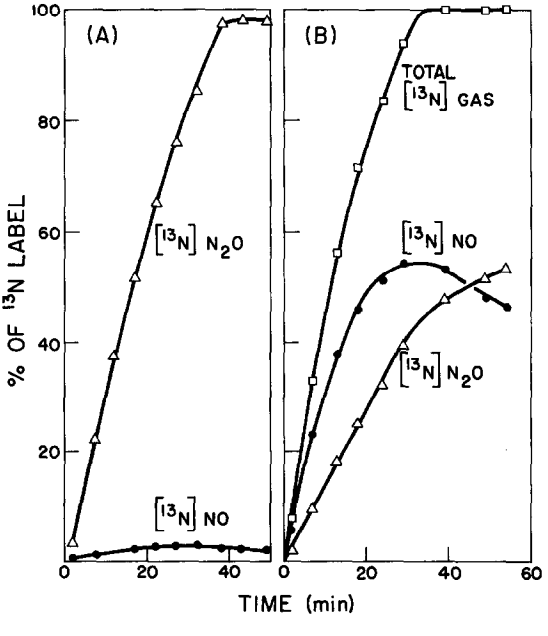

Figure 1. [^{13}N] gas production with time by P. aureofaciens (7.5 mg dry cell mass).
A. Quantity of nonlabeled NO added was 0.4 x 10^{-3} atm.
B. Quantity of nonlabeled NO added was 7.2 x 10^{-3} atm.

Table 2. Rates of ^{13}N gas production and consumption by P. aureofaciens.

Rate of:	Rate (nmole N per min)[a]	r^2[b]
In the presence of 7.2×10^{-3} atm NO		
Initial rates:		
$[^{13}N]NO$ production (0-7)[c]	44.8	0.998
$[^{13}N]N_2O$ production (0-13)	19.1	0.998
$[^{13}N]$Gas (total) (0-7)	64.1	0.997
Rates after NO_2^- depletion:		
$[^{13}N]NO$ consumption (39-54)	6.0	0.988
$[^{13}N]N_2O$ production (39-54)	5.0	0.999
In the absence of added NO		
Initial rates:		
$[^{13}N]N_2O$ production (2-12)	63.9	0.998

[a]All rates are based on the initial specific activity of NO_2^-. The expression of rates as "nmole N per min" means that rates of N_2O production (as nmole N_2O per min) are one-half those given in the table.

[b]Coefficients of determination.

[c]Period (min) of rate determined.

to the experiments reported here, in which the liquid phase was periodically sampled and analyzed for NO_2^-, indicated that the termination of $[^{13}N]$ gas accumulation coincided with depletion of added NO_2^-. The amount of label as $[^{13}N]NO$ began to decline at about the same time that $[^{13}N]NO_2^-$ was depleted. In the absence of added NO, essentially no $[^{13}N]NO$ was detected for P. aureofaciens.

Rates of $[^{13}N]$ gas appearance and disappearance were determined by linear regression and are given in Table 2. During the early portions of the experiments, rates of gas production were linear. The rate of total $[^{13}N]$ gas production in the presence of added NO, 64.1 nmole N per min, was the same as the rate of $[^{13}N]N_2O$ production in the absence of added NO (63.9 nmole N per min). After the $[^{13}N]NO_2^-$ was depleted, the rates of $[^{13}N]NO$ reduction and $[^{13}N]N_2O$ production were quite similar (6.0 and 5.0 nmole N per min, respectively).

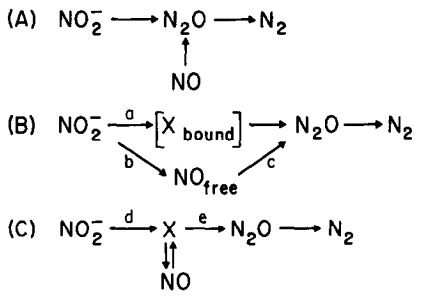

Figure 2. Suggested pathways of denitrification.
A. Proposed by St. John and Hollocher (2).
B. Proposed by Zumft (3).
C. Similar to that discussed by Payne (1).

DISCUSSION:

Interpretation of the data presented here can be facilitated by consideration of the previously proposed pathways for denitrification shown in Fig. 2. The scheme shown in Fig. 2A was proposed by St. John and Hollocher (2) as consistent with ^{15}N studies in P. aeruginosa. In this pathway NO is not an intermediate in reduction of NO_2^- to N_2O, but NO can be reduced to N_2O by a separate reductase. In the second pathway, Fig. 2B, Zumft (3) proposed that NO_2^- can be reduced to N_2O by two distinct routes. In one, the direct reduction of NO_2^- to N_2O (via X_{bound}) is coupled to phosphorylation while the alternate pathway provides "a means of discharging surplus of reductant" and includes the formation of a "free" NO intermediate. The third pathway shown (Fig. 2C) includes an unknown "X" intermediate with which NO is in equilibrium. If "X" is NO, then this is simply the pathway discussed by Payne (1).

In the experiments reported here, labeled N from $[^{13}N]NO_2^-$ exchanged with or accumulated in an added NO pool and the amount of ^{13}N appearing as $[^{13}N]NO$ increased with increasing additions of nonlabeled NO. This indicates that NO is produced during denitrification of NO_2^- and is not consistent with the pathway shown in Fig. 2A. These data may be explained by the schemes in Fig. 2B or C. However, when P. aureofaciens was incubated with 7.2×10^{-3} atm of added NO, the rate of $[^{13}N]NO$ accumulation (44.8 nmoles N per min) was more than twice the rate of $[^{13}N]N_2O$ production (19.1 nmoles N per min or 9.5

nmoles N_2O per min). If two alternate routes of NO_2^- reduction to N_2O exist in P. aureofaciens (as in Fig. 2B), the data reported here indicate that reduction of NO_2^- via NO is the dominant route.

The observation that the rate of total ^{13}N gas production ($[^{13}N]NO$ plus $[^{13}N]N_2O$) in the presence of a nonlabeled NO pool is the same as the rate of $[^{13}N]N_2O$ production in the absence of added NO indicates that the addition of NO does not alter the rate of NO_2^- reduction to gaseous product. These data also suggest that the rate limiting step in the reduction of NO_2^- to N_2O preceeds the formation of NO, that is, in Fig. 2B and C, the rate limiting steps are b and d relative to c and e respectively. This conclusion is not inconsistent with the rate of $[^{13}N]NO$ reduction to $[^{13}N]N_2O$ determined after the depletion of NO_2^-. The rate of NO reduction reported in Table 2 is based on the specific activities of the initial NO_2^- substrate. In the pool dilution experiments, the specific activity of the NO component was significantly reduced by the added nonlabeled NO. Thus, the actual rate of NO reduction to N_2O was much greater than the 6 nmoles N per min reported. However, the interpretation of these results is complicated by the possibility of an increase in the rate of NO reduction in the presence of added NO, and by the probability that the rate limiting step in the utilization of gaseous NO is the gas-liquid phase transfer, not the enzymatic reduction.

The data reported here appear to contradict the work of St. John and Hollocher with P. aeruginosa (2), in which exchange of the ^{15}N label from $[^{15}N]NO_2^-$ with a nonlabeled NO pool was not observed and little isotopic scrambling was found in the dinitrogen product derived from nonlabeled NO and $[^{15}N]NO_2^-$. The disparity in results may be due to the use of different organisms. However, the freedom of exchange of an intermediate may be a function not only of the concentration of the soluble nonlabeled pool (this work, 10) but also of the rate of N reduction (11). In the previous ^{15}N studies, the initial concentration of nonlabeled NO in solution was greater than in our ^{13}N studies (82 μM vs. a maximum of 14 μM); but the concentration of labeled

NO_2^- employed in the ^{15}N studies and the rate of its reduction were roughly 100 times greater than in the ^{13}N studies. The rate of N reduction may have been so high in the ^{15}N studies, that the concentration of nonlabeled NO in solution could not be maintained at or near the initial 82 μM by NO transfer from the gas to the liquid phase.

The data reported here indicate that during denitrification an intermediate occurs between NO_2^- and N_2O which is in equilibrium with NO. Whether this intermediate is actually NO and whether this route is the only means of NO_2^- reduction to N_2O are not yet clear.

ACKNOWLEDGEMENTS: We thank Dr. Michael Betlach for helpful discussions. This work was supported by NSF Grants DEB-77-19273 and PHY-78-01684 and USDA Regional Project NE-39.

REFERENCES:

1. Payne, W. J. (1973) Bacterial Rev., 37, 409-452.
2. St. John, R. T. and Hollocher, T. C. (1977) J. Biol. Chem., 252, 212-218.
3. Zumft, W. G. (1979) Naturwissenchaften, 66, 81-88.
4. Delwiche, C. C. and Bryan, B. A. (1976) Ann. Rev. Microbiol., 30, 241-262.
5. Stanier, R. Y., Palleroni, N. J. and Doudoroff, M. (1966) J. Gen. Microbiol. 43, 159-271.
6. Firestone, M. K. and Tiedje, J. M. (1979) Appl. Environ. Microbiol., 37, 673-679.
7. Tiedje, J. M., Firestone, R. B., Firestone, M. K., Betlach, M. R., Smith, M. S. and Caskey, W. H. (1979) Soil Sci. Soc. Am. J., 43, 709-716.
8. McElfresh, M. W., Meeks, J. C., and Parks, N. J. (1979) J. Radioanal. Chem., in press.
9. Wilhelm, W., Battino, R. and Wilcock, R. J. (1977) Chem. Rev. 77, 219-262.
10. Firestone, M. K., Ph.D. Dissertation, E. Lansing, Mich. (1978).
11. Betlach, M. R. and Tiedje, J. M., Manuscript in preparation.