**Communication**

H$_2^{18}$O Isotope Exchange Studies on the Mechanism of Reduction of Nitric Oxide and Nitrite to Nitrous Oxide by Denitrifying Bacteria

**EVIDENCE FOR AN ELECTROPHILIC NITROSYL DURING REDUCTION OF NITRIC OXIDE***

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Reduction of NO and NO$_2$ by whole cells of eight strains of denitrifying bacteria known to contain either heme cd$_1$, or copper-containing nitrite reductases (NiRs) has been examined in the presence of H$_2^{18}$O. All organisms containing heme cd$_1$, NiRs exhibited relatively large extents of exchange between NO$_2$ and H$_2^{18}$O (39–100%), as monitored by the $^{18}$O content of product N$_2$O. Organisms containing copper NiRs gave highly variable results, with *Achromobacter cycloclastes* and *Pseudomonas aureofaciens* exhibiting no $^{18}$O incorporation and *Rhodopseudomonas sphaeroides* and *Alcaligenes entrophus* exhibiting complete exchange between NO$_2$ and H$_2^{18}$O. Organisms containing heme cd$_1$, NiRs exhibited significant but lower levels of exchange between NO$_2$ and H$_2^{18}$O than between NO$_2$ and H$_2^{18}$O, while organisms containing copper NiRs gave significantly higher amounts of $^{18}$O incorporation than observed for the heme cd$_1$ organisms. These results demonstrate the existence of an NO-derived species capable of undergoing O-atom exchange with H$_2^{18}$O during the reduction of NO. Trapping experiments with $^{18}$O, $^{14}$N$_2$, and crude extracts of *R. sphaeroides* support the electrophilic nature of this intermediate and suggest its formulation as an enzyme nitrosyl, E-NO$^+$, analogous to that observed during the reduction of nitric oxide reductase (Equation 1, where E is the enzyme).

$$\text{NO}_2^{-} \rightarrow \text{NO}^{-} \xrightarrow{\text{NiR}} \text{NO}^{+} \xrightarrow{\text{NO}} \text{N}_2$$

The mechanism of microbial denitrification remains a controversial subject, despite a wealth of detailed studies on both intact bacteria and isolated enzymes (1–4). It is now generally accepted that denitrifying bacteria possess a nitric oxide reductase activity that is distinct from the nitrite reductase (NiR$^+$) activity. The latter are typically soluble enzymes that are rather easily purified and have been shown to be of two distinct types: a cytochrome cd$_1$-containing dimer of ~60-kDa subunits and a copper-containing enzyme that is more variable in both subunit size and degree of oligomerization (1, 5). The membrane-bound nature of the nitric oxide reductase activities has hindered their purification and characterization, but recently nitric oxide reductases have been purified to apparent homogeneity from two organisms (6–8) and shown to contain both heme b and c prosthetic groups (7, 8).

Virtually all workers in the field now agree that at least a significant portion of the total nitrogen flux occurs via a stepwise pathway with NO as an intermediate (Equation 1, where NO is nitric oxide reductase),

$$\text{NO}_2^{-} \rightarrow \text{NO}^{-} \xrightarrow{\text{NiR}} \text{NO}^{+} \xrightarrow{\text{NO}} \text{N}_2$$

Indeed, quantitative studies of NO levels during denitrification by several denitrifiers have been interpreted as indicating that only the former pathway (Equation 1) is operative and that NO is a free obligatory intermediate in denitrification (10–13). This conclusion is consistent with the observed lack of reduction of NO by isolated NiRs, with the fact that most isolated NiRs produce predominantly NO upon reduction of nitrite, and with the fact that mutants lacking either the heme cd$_1$ (14) or copper NiRs$^+$ are still capable of reducing NO. It fails, however, to account for the following observations. (i) Purified NiRs do, in at least some cases, produce significant amounts of N$_2$O that cannot be attributed to chemical reduction (15–17); (ii) NO$_2$ and reagents such as N$_2$ and H$_2^{18}$O that are known to react with a nitrosyl intermediate derived from NO$^+$ exhibit apparent competitive behavior (18, 19); (iii) the $^{18}$O content of N$_2$O produced from nitrite is about half that of nitrosation products derived from the nitrosyl intermediate, suggesting that unlabelled oxygen from a second nitrite enters the reaction (19); and (iv) the magnitude of the $^{18}$O isotope effect increases with increasing nitrite concentration, suggesting that two nitrite ions react with the enzyme prior to the first irreversible step (20, 21). Consequently, the relative importance of the routes shown in Equations 1 and 2 remains unclear.

In this communication, we present evidence that the NO reductase exhibits a remarkable and previously unsuspected similarity to the NiRs in that an electrophilic species derived from NO can be trapped during the reduction of NO. Further,
we demonstrate via comparison of the amount of $^{15}$O incorporated into $N_2$O from either NO$_2^-$ or NO that, in certain bacteria at least, reduction of NO$_2$ may not proceed entirely according to the stepwise pathway shown in Equation 1.

**MATERIALS AND METHODS**

**Bacterial Strains—**Pseudomonas aeruginosa PAO1 was from B. W. Holloway, Monash University, Clayton, Australia; Rhodopseudomonas sphaeroides forma sp. denitrificans was from T. Satoh; and Pseudomonas fluorescens AK-15 was a soil isolate obtained in this laboratory. The rest of the strains used were from ATCC. Characterization of copper-type or cytochrome cd$_3$ nitrite reductases in some of these strains has been described (22); classification of Alcaligenes eutrophus ATCC 17699 as a copper N Ri-containing organism was performed using N,N-dimethyldithiocarbamate as described (22).

**Sample Preparation—** Cultures were grown anaerobically in 3% acetylene (0.2 atmosphere) was added to the gas phase to block nitrous oxide reductase activity. Cell density corresponded to a protein concentration of 11 mg/ml. 1.0 pmol of NO or nitrite was used as substrate. H$_2^{18}$O was present at the range of 8-13%. After completion of the reaction (50 min), 0.1 ml of 10 N NaOH was added to the reaction vial. Data are means of three replicates from two independent experiments.

**RESULTS AND DISCUSSION**

**Exchange with H$_2^{18}$O during Nitrate Reduction—**The results obtained for four denitrifiers known to contain heme cd$_3$ NiRs and four denitrifiers known to contain copper NiRs are presented in Table I, column 1. As expected based upon previous work demonstrating the existence of an electrophilic nitrosyl intermediate during reduction of nitrite by the heme cd$_3$ NiRs (18, 19, 23-25), organisms containing such enzymes exhibited relatively large amounts of $^{15}$O incorporation from H$_2^{18}$O (extents of exchange ranging from 39 to 80%) during reduction of NO$_2^-$ to N$_2$. In fact, strain 2 exhibited significantly more than 50% exchange. This is significant because our original hypothesis regarding the direct pathway (9) postulated that the N-N bond was formed by attack of a second NO$_2^-$ upon an electrophilic nitrosyl intermediate, E-N-NO$_2^-$, derived from the first nitrite, which is known to undergo facile $^{15}$O exchange by a reversible hydration/dehydration process (23-25). Shearer and Kohl (21) have shown that the NiR from P. stutzeri JM300 is a "sticky" enzyme and that NO$_2^-$ is committed to reduction once bound. Hence, $^{15}$O-labeled NO$_2^-$ does not accumulate and $^{15}$O incorporations of >50% are not consistent with the direct pathway (Equation 2), unless the NiR in organism 2 differs substantially from that in P. stutzeri JM300 (organism 3).

The organisms containing copper NiRs exhibited significantly different behavior in two cases (6 and 7), where essentially no $^{15}$O incorporation into N$_2$O product was observed. This result is consistent with previous work on the Achromobacter cycloclastes system, which showed undetectable amounts of $^{15}$O exchange (26). In contrast, *R. sphaeroides* exhibited virtually complete exchange between H$_2^{18}$O and product N$_2$. Suggesting the presence of an electrophilic nitrosyl intermediate in these organisms that differs dramatically in its reactivity with H$_2^{18}$O from the other organisms containing a copper NiR. Such behavior is perhaps not surprising, given the extreme variability in subunit size and immunoreactivity observed with copper NiR-containing organisms (22).

**Exchange with H$_2^{18}$O during Reduction of NO—**As shown in column 2 of Table II, substantial amounts of $^{15}$O were also observed in N$_2$O produced by reduction of NO. The extent of $^{15}$O incorporation observed with NO tended to be lower than that observed with NO$_2^-$ for the same organism (except for 6 and 7, see below) but was well above background levels for all but one case. The four organisms with copper NiRs gave a significant amount of exchange above background levels (Table II).

**TABLE I**

| Strain | Substrate | Nitrite | NO |
|--------|-----------|---------|----|
| 1. *P. denitrificans* ATCC 19367 | 59 ± 5 | 11.3 ± 1.2 |
| 2. *P. aeruginosa* PAO1 | 76 ± 7 | 19.0 ± 0.2 |
| 3. *P. stutzeri* JM300 | 58 ± 14 | 4 ± 1 |
| 4. *P. fluorescens* AK-15 | 39 ± 1 | 15.0 ± 1.2 |
| 5. *A. eutrophus* ATCC 17699 | 94 ± 5 | 84 ± 6 |
| 6. *A. cycloclastes* ATCC 21921 | 4 ± 2 | 30 ± 7 |
| 7. *P. aureofaciens* ATCC 13985 | 6.0 ± 0.2 | 37 ± 14 |
| 8. *R. sphaeroides* forma sp. denitrificans | 90 ± 15 | 61 ± 19 |

**TABLE II**

| Sample | N$_2^{15}$O | N$_2^{18}$O | Exchange |
|--------|-------------|-------------|-----------|
| Crude extract | 23.0 ± 0.3 | 477 ± 0.3 | 100 |
| Crude extract + PMS (40 μM) | 23.2 ± 0.3 | 477 ± 0.3 | 100 |
| Crude extract + NADH (4 mM) | 13 ± 1.2 | 487 ± 1 | 56 |
| Crude extract + NADH (4 mM) + PMS (40 μM) | 3.2 ± 0.3 | 497 ± 0.3 | 9 |

**TABLE III**

| Isotope | Amount | Exchange | Nitrosation |
|---------|--------|---------|-------------|
| 14,15$N_2^{15}$O | 0.74, 0.88 | % | % |
| 14,15$N_2^{18}$O | 20, 24 | 80, 79 | 13, 14 |
| 15$N_2^{15}$O | 5.1, 5.8 | 82, 87 |
| 15$N_2^{18}$O | 134, 136 | 82, 87 |

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3 A. Arunakumari and J. M. Tiedje, unpublished results.
Mechanism of Reduction of NO and Nitrite by Denitrifiers

\[
E + {^{15}NO} \rightarrow E^{15}NO^{+} \rightarrow E^{15}NO^{+} + {^{18}O_2} \rightarrow E^{15}NO^{+} \rightarrow E + {^{15}NO}
\]

Scheme 1

\[
E + {^{15}NO} \rightarrow E^{15}NO^{+} \rightarrow E^{15}NO^{+} + {^{18}O_2} \rightarrow E^{15}NO^{+} \rightarrow E + {^{15}NO}
\]

Scheme 2

significantly higher extents of \(^{18}O\) exchange (30–84%) than did those known to contain a heme cd\(_1\) NiR (4–19%), but the origin of this difference is unclear since an NO reductase has yet to be purified from any of the former.

The data in Table I, column 2, demonstrate the presence of an NO-derived species capable of undergoing O-atom exchange with H\(_2\)\(^{18}O\) during the reduction of NO, which would not be expected \textit{a priori} to proceed via an electrophilic species. The overall reaction can be written as

\[
2H^+ + 2e^- + 2NO \rightarrow N_2O + H_2O
\]

indicating that water is produced during the reaction (presumably by protonation and dehydration of a hyponitrite level species containing two N atoms, such as N\(_2\)O\(_5\)), suggesting a possible route for \(^{18}O\) incorporation if the final dehydration step were reversible. As a control experiment, cells of \textit{Pseudomonas aerofaciens} were grown on N\(_2\)O, suspended in medium containing 10\% H\(_2\)\(^{18}O\), and incubated anaerobically for 5 h at room temperature with 0.1 ml of N\(_2\)O (8.8 \(\mu\)mol) in an 8-ml bottle. The \(^{18}O\) content of the N\(_2\)O was measured and did not differ from the natural abundance. Thus, the observed \(^{18}O\) incorporation during reduction of NO must occur \textit{prior} to reduction to the N\(_2\)O level.

This conclusion is also supported by the data in Table II, which demonstrate that for \textit{R. sphaeroides} at least the extent of exchange with H\(_2\)\(^{18}O\) decreased as the concentration of electron donor/mediator was increased. This suggests strongly that \(^{18}O\) exchange occurs via a relatively oxidized nitrogen intermediate.

\textbf{Trapping with Azide during NO Reduction}—If an electrophilic NO-derived species is indeed present during reduction of NO, it might be expected to react with nucleophiles other than H\(_2\)\(^{18}O\). For example, N\(_2\) and NH\(_2\)OH have been reported to trap the electrophilic nitrosyl produced by the heme cd\(_1\) NiRs (19, 20, 23–25). Thus, crude extracts of \textit{R. sphaeroides} were treated with \(^{15}NO\) in the presence of \(^{15}N_2\) and H\(_2\)\(^{18}O\); the amounts of the various isotopically labeled forms of N\(_2\)O formed are given in Table III. It is clear that substantial (\textasciitilde 14\%) amounts of a nitrosation product \(^{15}N_2\)O were observed even at the relatively low azide concentration used (1 mM) and that both the nitrosation product and NO reduction product exhibited comparable \(^{18}O\) incorporation.

\textbf{Implications for the Mechanism of NO Reduction}—The H\(_2\)\(^{18}O\) exchange and N\(_2\) trapping results presented above strongly imply the presence of an electrophilic mononitrogen intermediate during reduction of NO. The simplest such species is an enzyme nitrosyl, E-NO\(_2\), analogous to that observed for the heme cd\(_1\) NiRs. This is a most unexpected result, since it is not obvious why an oxidized NO species should be an intermediate in its reduction to N\(_2\)O. We note that similar results observed earlier for \textit{P. stutzeri JM} 300 in the presence of NO and H\(_2\)\(^{18}O\) or \(^{15}NO\) and \(^14NH_2OH\) (19) are also completely consistent with the results reported here (although they were initially attributed to an NO complex of the heme cd\(_1\) NiR in this organism).

Two possible tentative explanations for the data are shown in Schemes 1 and 2. In Scheme 1, the \(^{18}O\) exchange and nucleophilic trapping occur via a species that is not on the catalytic pathway for NO reduction but is rather on an oxidized “shunt.” Given the redox potentials for synthetic heme nitrosyls (27–29), it might not be surprising if electron transfer to another center on the enzyme or elsewhere were to generate a transient oxidized species that, as shown, has nothing to do with catalysis. The other extreme is represented in Scheme 2, in which the E-NO\(_2\) species is an obligatory intermediate, possibly reacting via a hypothetical enzyme-bound NO\(_2\) with a second E-NO\(_2\) in a fashion analogous to that postulated by us earlier for the reduction of NO\(_2\) by NiR (9). Available data do not permit us to distinguish between these alternatives or the many possible variants thereof.

\textbf{Implications for the Pathway of Denitrification}—Examination of the data in column 1 and 2 of Table I reveals that, in most cases, our results are fully compatible with NO as an obligatory intermediate in denitrification, i.e. the sequential pathway shown in Equation 1. That is, for organisms 1–5 and 6 and 7, the extent of \(^{18}O\) incorporation observed for NO; as substrate is far less than with NO. (If the situation shown in Equation 1 were to obtain, the amount of \(^{18}O\) incorporated into N\(_2\)O derived from NO\(_2\) would always have to be at least equal to that in N\(_2\)O derived from NO.) Thus, for these organisms at least, the sequential pathway of Equation 1 appears to be significantly less important than a direct pathway as indicated in Equation 2.

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