Evidence from Isotope Labeling Studies for a Sequential Mechanism for Dissimilatory Nitrite Reduction

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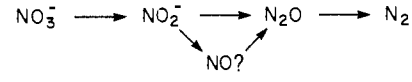
Abstract: Results from three independent experimental approaches using stable isotopes (^{15}N or ^{18}O) and crude cell-free extracts of *Pseudomonas stutzeri* are presented as evidence for a sequential mechanism for nitrite reduction in denitrification. (i) Competition between nitrosation and denitrification reactions catalyzed by nitrite reductase has been demonstrated, indicating that NO_2^- and N_3^- both compete for a common nitrosyl intermediate. (ii) The ^{18}O enrichment of the denitrification product (N_2O-46 vs N_2O-48) was significantly lower than that of the nitrosation product (N_2O-45 vs N_2O-47) when both reactions were carried out simultaneously in ^{18}O-labeled water with $^{15}NO_2^-$ as substrate and $^{14}N_3^-$ as nucleophile, indicating that the denitrification product contained oxygen that did not originate from the nitrosyl intermediate. (iii) The extent of isotopic equilibration between $H_2{}^{18}O$ and denitrification product (N_2O) decreased as the concentration of nitrite increased, demonstrating that $H_2{}^{18}O$ and NO_2^- compete for a common nitrosyl intermediate (Aerssens, E.; Tiedje, J. M.; Averill, B. A. *J. Biol. Chem.* **1986**, *261*, 9652-9656). Taken together, the results demonstrate the validity of our proposed pathway (Averill, B. A.; Tiedje, J. M. *FEBS Lett.* **1982**, *138*, 8-11) for nitrite reduction, in which the N-N bond of N_2O is formed by nucleophilic addition of nitrite to an enzyme-bound nitrosyl intermediate. Removal of the nitrite reductase from its membrane environment apparently results in a perturbation of the in vivo reaction kinetics, suggesting that the fate of the nitrosyl intermediate is altered in the solubilized enzyme. This could explain some of the variability in the reaction products ratio (NO/N_2O) found for various enzyme preparations under different conditions, and in particular, it may account for the observation that purified nitrite reductases produce mainly NO rather than N_2O.

The pathway by which denitrifying bacteria convert nitrate to N_2 consists of at least three distinct enzyme-catalyzed steps, as shown in Scheme I. The first step is catalyzed by a molybdenum-containing dissimilatory nitrate reductase,[1] while the last step is catalyzed by an unusual copper-containing reductase.[2] In contrast, the second step has remained controversial, with disagreement as to whether it is carried out by a single enzyme or by two enzymes, with NO as a free obligatory intermediate. Even among workers who favor the single enzyme interpretation, there is substantial controversy[3] regarding the mechanism by which two nitrite ions are converted to N_2O: (i) is the N-N bond of N_2O formed by nucleophilic attack of a second NO_2^- upon a metal-coordinated nitrosyl species[4] (Scheme II), or (ii) is the nitrosyl intermediate first reduced to free nitroxyl (HNO), which spontaneously dimerizes to N_2O[5] (Scheme III)?

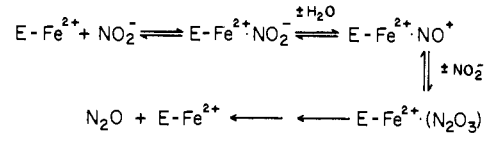
Although studies in a number of laboratories have been interpreted as favoring NO as a free obligatory intermediate (thus implying the existence of a separate NO reductase),[3,6,7] definitive evidence is still lacking. It has, however, been conclusively demonstrated by $H_2{}^{18}O$ exchange and trapping experiments that nitrite reduction by whole cells[8] and the purified heme cd_1-containing nitrite reductase[5] of *Pseudomonas aeruginosa* proceeds via an enzyme-bound nitrosyl intermediate (E-NO^+). This nitrosyl intermediate arises from dehydration of coordinated nitrite[5,8] and is common to Schemes II and III (and possibly to NO formation from NO_2^- as well). Intermediates beyond the nitrosyl species remain nebulous, although evidence against the intermediacy of *trans*-hyponitrite ($N_2O_2^{2-}$)[9] and oxyhyponitrite ($N_2O_3^{2-}$)[10] has been presented. The demonstration of positional isotopic equivalence of nitrogen atoms in product N_2O[11] was interpreted as favoring HNO as an intermediate (Scheme III), but in fact coordinated *cis*-hyponitrite is equally plausible if it undergoes a rapid intermolecular exchange of the coordinated and uncoordinated nitrogen atoms, as has been observed for a related system.[12]

Thus, with the possible exception of the oxyhyponitrite study,[10] no evidence that would unambiguously distinguish among the three possible mechanisms has been reported. Competition between denitrification and nitrosation reactions has been demonstrated

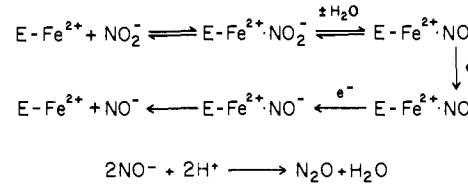
Scheme I. Pathway of Denitrification, with Identified or Postulated Intermediates Indicated



Scheme II. Formation of the N-N Bond by Nucleophilic Attack of Nitrite on an Enzyme-Bound Nitrosyl



Scheme III. Formation of Nitrous Oxide by Dimerization of Nitroxyl Anions



only with ^{15}NO and NH_2OH, suggesting that an enzyme–nitrosyl intermediate is common to both reactions when NO, rather than

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NO_2^-, is the substrate;[13] the relevance of this finding to nitrite reduction is perhaps open to question. The only experiments that have provided evidence, albeit indirect, for Scheme III vs Scheme II are studies on the ^{15}N isotope effect associated with denitrification of nitrite by *P. stutzeri* cells[14] and in soils,[15] which found that the magnitude of the isotope effect increased with increasing nitrite concentration. The most obvious (but not unique) interpretation is that this result is due to sequential addition of two nitrite ions to the enzyme prior to the first irreversible step.

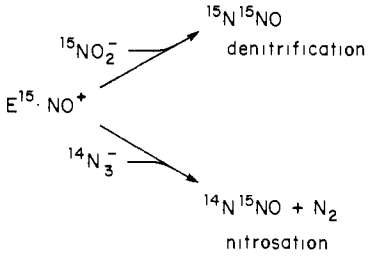
In a previous paper,[13] we presented our first studies on the pathway of dissimilatory nitrite reduction. With whole cells of *P. stutzeri*, we found that the extent of isotopic equilibration between $H_2{}^{18}O$ and the product of dentrification, N_2O, decreased as the nitrite concentration increased, suggesting that $H_2{}^{18}O$ and NO_2^- compete for a common intermediate (i.e., favoring Scheme II). In this work, we assumed that the ^{18}O enrichment of the free nitrite pool was negligible during the time of our measurements; this has now been confirmed experimentally by Shearer and Kohl.[16] *P. stutzeri* nitrite reductase is thus a "sticky" enzyme, meaning that nitrite, once bound to the enzyme, is committed to react and does not readily dissociate.

In this paper, we present the results of stable isotope studies focused on elucidating the pathway of nitrite reduction and on the effect of solubilization of the enzyme on the relative rates of the individual steps. We present evidence that strongly favors the pathway represented in Scheme II: N_3^-, $H_2{}^{18}O$, and NO_2^- all compete for a common enzyme-bound nitrosyl intermediate. This implies that the N–N bond of N_2O must be formed via nucleophilic attack of a second nitrite ion on a coordinated nitrosyl derived from the first nitrite. Our results are inconsistent with a pathway such as the one represented in Scheme III, in which two equivalent nitroxyl anions combine to form nitrous oxide,[5] and also with NO as a free intermediate.

Experimental Section

Cell Growth and Assay Conditions. The bacterial strain used was *P. stutzeri* JM 300. Cultures were grown and harvested as described earlier,[13] but cells were washed only once instead of three times. Cells were resuspended in a volume of 50 mM HEPES (*N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid) buffer, pH 7.3 equal to 1% of the harvested volume. Cell disruption was by French press (3 passages, 12 000 psi) or by sonication with a Branson sonifier (2.5 min on ice, 5 × 30 s at 40% of maximum output with 30-s intervals) as specified below for each experiment. Cell-free crude extracts were obtained by filtration through a 0.22-μm filter. These extracts contained no whole cells, as they were unable to initiate growth when inoculated into sterile medium, even after several weeks of monitoring for possible growth. Typical assay conditions are given here; variations are indicated in the text. Assays were carried out in 8-mL serum vials sealed with butyl rubber stoppers and aluminum crimps. They contained 1 or 3 mL of liquid phase. The final buffer concentration in the assay mixtures was 25 mM HEPES (pH 7.3). The reducing system used was sodium succinate and the natural electron transport components present in the cell-free crude extracts. The reaction was initiated by anaerobic addition of nitrite. Labeled water ($H_2{}^{18}O$) was added to the buffer. Addition of acetylene to inhibit nitrous oxide reductase proved unnecessary since this enzymatic activity virtually disappeared upon cell disruption. Vials were made anaerobic by flushing with argon. For each newly prepared extract, the specific activity was established by measurement of nitrous oxide production on a gas chromatograph. The incubation periods were chosen such that the extent of reaction, measured by GC and expressed as N_2O produced as a fraction of the maximum N_2O possible, would never exceed 20%. This was crucial for the nitrosation experiments, where it was assumed that the nitrite and azide concentrations were roughly constant during the course of the experiment. Samples were analyzed by GC/MS immediately or stored frozen for later analysis. Injection volumes were chosen to maintain a roughly constant number of counts of total N_2O, for maximum accuracy in determining ratios of isotopically labeled species. The rate of N_2O production from 1 mM nitrite plus 50 mM azide was about 0.01 mM N/h. Assays contained 0.1–0.3 mL of crude cell-free extract. Denitrification rates in crude extracts were 2 orders of magnitude lower than in whole cells. All experiments were reproduced at least twice.

Scheme IV. Nitrosation and Denitrification Products from $^{15}NO_2^-$ and N_3^-



Reagents. All chemicals used were reagent grade. Azide stock solutions were prepared immediately before each use. Stable isotopes were obtained from Monsanto (Mound, OH). The ^{18}O-labeled water contained 15 atom % ^{18}O. Isotopic purity of the ^{15}N-labeled nitrite was better than 99.9%.

Controls. Sterile controls (autoclaved for 20 min at 121 °C) and controls without isotope were routinely performed for each experiment. Experiments were designed so that chemical N_2O production was negligible.

GC/MS Equipment and Conditions. We used both an HP 5985 and an HP 5995 GC/MS; the latter gave much better sensitivity. Source and mass analyzer temperatures were set at 150 °C to prevent excessive decomposition of N_2O to N_2 and NO in the instrument (Weeg-Aerssens, E., unpublished observations). The electron multipliers were set in the range 200–1000 eV above autotune.

Results and Discussion

Competition between Denitrification and Nitrosation. In our earlier work, we examined the competition beween denitrification and nitrosation reactions using whole cells of *P. stutzeri* and NH_2OH as a nucleophile to trap the nitrosyl intermediate. We found that hydroxylamine was relatively inefficient at trapping the nitrosyl species, giving only a few percent nitrosation product at 10–320 mM NH_2OH and 0.1 mM NO_2^-.[13] More importantly, however, we found that there was evidence for ^{18}O exchange into an intermediate in the nitrosation reaction that is downstream from the enzyme–nitrosyl complex.

Accordingly, we have examined azide as the trapping nucleophile. Since it cannot form an N_2O_2 intermediate during nitrosation, it decreases the probability of ^{18}O exchange into subsequent nitrosation intermediates. Azide was, however, completely ineffective as a nitrosyl trapping agent with whole cells under the conditions examined (1 mM $Na^{15}NO_2$, 1–10 mM N_3^-, fresh LB medium as reductant); insignificant amounts of nitrosation products (≤1% of total N_2O) were observed. Since azide is known to trap the nitrosyl intermediate in crude cell-free extracts[8] and in the purified heme cd_1 enzymes,[5] this suggests that azide is not readily transported through the cell membrane. Indeed, we found that cell-free extracts prepared by French press treatment showed up to 30% nitrosation with 50 mM NaN_3 and 0.1–1.0 mM $Na^{15}NO_2$.

If nitrite and a nucleophile such as azide are indeed competing for the introsyl intermediate common to Schemes II and III, then it is possible to quantitate the flux through denitrification vs nitrosation by measuring the relative amounts of N_2O-46 and N_2O-45, respectively (Scheme IV). Thus, the competition can be examined as a function of nitrite or azide concentration. Systematically varying the N_3^- concentration at constant $[NO_2^-]$ will not distinguish the pathways of Schemes II and III, since in either case the amount of nitrosation is expected to increase with increasing $[N_3^-]$. This type of experiment would simply confirm the existence of a trappable nitrosyl intermediate.[5,8] In contrast, if the $[NO_2^-]$ is varied at constant $[N_3^-]$, the predicted results differ for the two schemes. For the pathway in Scheme II, the

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Table I. Ratio of Nitrosation (N_2O-45) to Denitrification (N_2O-46) Products as a Function of Nitrite Concentration.[a]

$Na^{15}NO_2$, mM	N_2O-45	N_2O-46	$\frac{N_2O\text{-}45}{N_2O\text{-}46}$
0.1	11440	46380	0.25
	11080	48430	0.23
			av 0.24 ± 0.01
0.2	11650	48080	0.24
	8865	41700	0.21
			av 0.22 ± 0.02
0.5	3800	43820	0.087
	6295	73710	0.085
			av 0.086 ± 0.001
1.0	3565	60220	0.059
	2320	49740	0.047
	2680	54170	0.050
			av 0.052 ± 0.005

[a] Numbers are integrator counts for a standard injection volume. Conditions: $[NaN_3]$ = 50 mM, reductant = Luria broth; cell-free crude extracts of *P. stutzeri* prepared via French press; two or three replicates per nitrite concentration, all shown. Background N_2O (m/z 45) was 1% of total N_2O in control samples without azide. No significant N_2O was formed in sterile controls.

Table II. Ratio of Nitrosation (N_2O-45) to Denitrification (N_2O-46) Products as a Function of Nitrite Concentration with Succinate as Reductant[a]

$Na^{15}NO_2$, mM	N_2O-45	N_2O-46	$\frac{N_2O\text{-}45}{N_2O\text{-}46}$
0.05	7110	9834	0.72
	8710	10080	0.86
			av 0.79 ± 0.07
0.10	9555	21500	0.44
	10350	22900	0.45
			av 0.44 ± 0.01
1.0	11313	56575	0.20
	11075	72925	0.15
			av 0.17 ± 0.03
10.0	11450	41500	0.28
	8913	39750	0.25
			av 0.27 ± 0.03

[a] Conditions as in Table I except for use of 50 mM sodium succinate in place of Luria broth medium.

Table III. Effect of Reductant on Ratio of Nitrosation to Denitrification Products[a]

	$\frac{N_2O\text{-}45}{N_2O\text{-}46}$	
succinate, mM	$[^{15}NO_2^-]$ = 10 mM	$[^{15}NO_2^-]$ = 0.05 mM
10	0.31 ± 0.03	0.57 ± 0.04
	0.59 ± 0.02*	
50	0.19 ± 0.02	0.49 ± 0.01
	0.25 ± 0.05*	
100	0.17 ± 0.01	0.54 ± 0.10
	0.17 ± 0.01*	

[a] Conditions as in Table II except for indicated concentrations of succinate. Data from repeated experiments using different cell preparations are indicated by an asterisk.

nitrosation:denitrification product ratio should decrease with increasing $[NO_2^-]$, since the second NO_2^- will compete with N_3^- for the nitrosyl intermediate. For a pathway such as that in Scheme III, the nitrosation:denitrification ratio should be independent of $[NO_2^-]$, since nitrite does not appear in the scheme after formation of the nitrosyl intermediate.

The data shown in Table I demonstrate that the N_2O-45/N_2O-46 ratio decreased with increasing $[NO_2^-]$, indicating that denitrification is indeed of a higher kinetic order with respect to nitrite than is nitrosation. At higher nitrite concentrations, a saturation effect was observed, as expected. Earlier attempts[5] to demonstrate this competition reaction failed, presumably because of the very high nitrite concentrations used (≥10 mM NO_2^-).

Effect of Reductant on the Nitrosation:Denitrification Ratio. In order to examine a more chemically defined system than LB medium, i.i which the actual reductant concentration is unclear, we carried out the same competition experiment as described above utilizing 50 mM sodium succinate as the reductant. The results are given in Table II. The same general trend was observed as with LB medium, namely that the ratio of N_2O-45 (arising from nitrosation) to N_2O-46 (arising from denitrification) decreased with increasing concentration of nitrite, at least for $[NO_2^-] \leq 1$ mM. The relative amount of nitrosation at a given nitrite concentration was, however, approximately a factor of 2–3 higher with succinate vs LB medium, for reasons that are not clear.

The fact that the trend in N_2O-45/N_2O-46 ratio reversed between 1 and 10 mM nitrite suggested that reductant had become rate-limiting for denitrification at the highest nitrite concentrations. Accordingly, we examined the effect of varying the succinate concentration at 0.05 and 10 mM NO_2^- (Table III). The data clearly show that the nitrosation:denitrification product ratio decreased with increasing succinate concentration at 10 mM NO_2^-. The variability between repeated experiments is relatively large for less than saturating levels of succinate, because the concentration of residual reductant from the growth medium varied, depending on how long the cells were starved for carbon prior to harvesting. Denitrification rates were independent of reductant at very low NO_2^- concentrations, as expected.

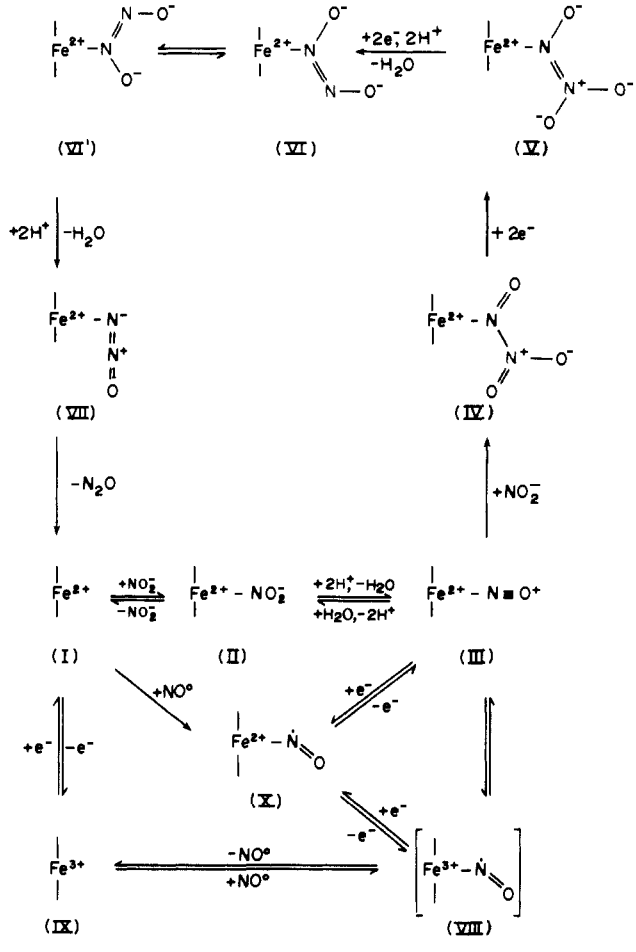
Oxygen-18 Content of Nitrous Oxide from Denitrification vs Nitrosation. The major difference between the two proposed mechanisms of denitrification (Schemes II and III) is the nature of the two nitrogen species that form the N–N bond. In Scheme III, the N–N bond is formed by reaction of two equivalent nitroxyl anions (NO^-). Therefore, if the reaction is carried out with $^{15}NO_2^-$ in the presence of $H_2{}^{18}O$, the ^{18}O content of the E–$^{15}NO^+$ intermediate will be reflected in the ^{18}O content of the denitrification product, $^{15}N_2O$, which can be measured directly. The ^{18}O content of the nitrosyl intermediate can be measured by trapping it with $^{14}N_3^-$, which yields $^{14}N^{15}NO$; the ^{18}O content of the nitrosation product will also be the same as that of the E–NO^+ intermediate. In Scheme II however, the two nitrogen species are not equivalent at the point at which the N–N bond is formed by attack of free NO_2^- on the E–NO^+ intermediate. Only the E–NO^+ reactant will contain appreciable amounts of ^{18}O. Free nitrite will be virtually unlabeled, since the rate for dissociation of NO_2^- from the enzyme is very low,[16] and any ^{18}O-labeled nitrite formed would be diluted by the nitrite pool. The results of experiments in which the ^{18}O content of N_2O originating from denitrification [N_2O-

Table IV. ^{18}O Isotopic Enrichment of Denitrification (N_2O-46 and N_2O-48) and Nitrosation (N_2O-45 and N_2O-47) Products[c]

N_2O-46	N_2O-48	% equilib	N_2O-45	N_2O-47	% equilib
11 807 400	620 200	53.2	8 702 400	776, 400	88.8[a]
13 823 800	838 500	51.2	9 364 600	760, 500	81.3[a]
		av 52.2 ± 4.0			av 85.1 ± 3.8
28 000	1235	55.0	3110	210	83.9[b]
25 380	1085	53.3	2600	160	76.6[b]
17 930	730	50.7	2810	180	79.7[b]
16 200	670	51.6	2280	150	81.8[b]
		av 52.7 ± 2.0			av 80.5 ± 2.4

[a] Data obtained on HP 5995 with 9 atom % $H_2{}^{18}O$. [b] Data obtained on HP 5985 with 7.3 atom % $H_2{}^{18}O$. [c] Numbers are integrator counts for a standard injection volume. Conditions: $[NaN_3]$ = 50 mM, $[Na^{15}NO_2]$ = 1.0 mM, reductant = 100 mM sodium succinate; cell-free extracts prepared by sonication.

Scheme V. A Detailed Representation of the Proposed Pathway by Which NO_2^- Is Reduced to N_2O via Sequential Reaction of Two NO_2^- Ions with the Enzyme



48/(N_2O-46 + N_2O-48)] and from nitrosation with N_3^- [N_2O-47/(N_2O-45 + N_2O-47)] was determined are shown in Table IV. We found that $^{15}N_2O$ from denitrification is 50%–60% equilibrated with the $H_2{}^{18}O$, while $^{14}N^{15}NO$ from nitrosation is 80–85% equilibrated with the $H_2{}^{18}O$. This is consistent only with Scheme II, in which nitrite containing no ^{18}O dilutes the ^{18}O content of the E–NO^+ intermediate.

The ratios of the extent of equilibration are not exactly 1:2, as predicted by Scheme II and in more detail in Scheme V, assuming that oxygen atoms are lost with equal probability from either nitrogen atom during conversion of the dinitrogen intermediates to N_2O. One possible explanation is the fact that, even at high [NO_2^-] in the absence of N_3^-, we always observe ca. 8% equilibration of N_2O with $H_2{}^{18}O$ (data not shown). This indicates either that NO_2^- is unable to completely suppress ^{18}O exchange into the E–NO^+ intermediate or that some ^{18}O exchange occurs via an intermediate containing an N–N bond, as we have observed for nitrosation with NH_2OH.[13]

Mechanistic Implications. The results presented above demonstrate that nitrite competes with azide for the E–NO^+ intermediate that is formed by dehydration of nitrite. Our earlier work[13] and the isotope dilution experiments described above demonstrate that nitrite competes with $H_2{}^{18}O$ (and with azide) for the same E–NO^+ intermediate. These results are consistent *only* with a sequential mechanism for reaction of two nitrite ions to form N_2O (e.g., a mechanism such as that outlined in Scheme II), and eliminate the nitroxyl mechanism (Scheme III). Because the mechanism of denitrification has been the object of substantial controversy, it is worthwhile to briefly consider the major lines of evidence adduced previously in favor of other mechanisms and against the sequential mechanism indicated by the present data.

To facilitate this, a more detailed version of the mechanism shown in Scheme II is presented in Scheme V; this is an expanded version of the hypothetical pathway presented in our original paper.[4] This pathway (upper portion of Scheme V) has several key features. Initially, nitrite binds to a ferrous heme (I) and is dehydrated to a reactive[17,18] ferrous–nitrosyl complex (III) via the ferrous–nitrite complex (II), as demonstrated by Hollocher and co-workers.[5,8] Nucleophilic attack of a second nitrite on the coordinated NO^+ of III produces IV, containing bound N_2O_3. Reduction by two electrons produces V, containing coordinated oxyhyponitrite, $N_2O_3^{2-}$. Reduction by a second two electrons and dehydration produces a species (VI) containing coordinated *cis*-hyponitrite, $N_2O_2^{2-}$, which upon further dehydration yields the ferrous–N_2O complex (VII). Loss of N_2O from VII regenerates the ferrous heme (I). The lower portion of this scheme shows how evidence previously interpreted as favoring NO as a free intermediate may be accounted for and is discussed below.

The major argument presented in favor of nitroxyl as an intermediate[8,11] has been the positional isotopic equivalent of nitrogen in $^{14,15}N_2O$ produced by concomitant reduction of $^{15}NO_2^-$ and ^{14}NO observed by Garber and Hollocher.[11] Their data argue for a symmetrical intermediate in the reaction, which could be *either* a free mononitrogen intermediate (e.g., HNO) *or* an effectively symmetrical dinitrogen intermediate. The latter is perfectly consistent with a sequential mechanism if the coordinated *cis*-hyponitrite intermediate (VI in Scheme V) interconverts rapidly between the two isomers with different nitrogen atoms coordinated to iron (VI ⇄ VI′). Available chemical evidence suggests that this equilibration is likely to be very rapid. For example, variable-temperature NMR studies have shown that substituted pyridazines (which also contain two sp^2 nitrogen atoms linked by a formal double bond) when bound to ruthenium porphyrins exchange nitrogen donor atoms in an intermolecular process at rates of 10^2–10^6 s^{-1}.[12] Since substitution reactions of ruthenium complexes are generally much slower than for the corresponding iron complexes, one would expect such reactions at iron to be very rapid indeed, much faster than the overall enzymatic reaction. Thus, both the data on positional isotopic equivalence[11] and the ^{18}O enrichments[8] reported by Garber and Hollocher are equally consistent with either the nitroxyl or sequential mechanism.

Scheme V postulates that oxyhyponitrite, $N_2O_3^{2-}$, is an enzyme-bound intermediate. Since $Na_2N_2O_3$ is readily prepared,[19] it is possible to examine whether $N_2O_3^{2-}$ is converted to N_2O by the enzyme. Experiments with several denitrifying bacteria and whole-cell extracts have been reported by Garber, Wehrli, and Hollocher as evidence that $N_2O_3^{2-}$ "can be neither a free nor an enzyme-bound intermediate" in denitrification.[10] This conclusion is open to question on two levels. First, the bacteria and extracts used showed very low denitrification activity (on the order of only 2-fold higher than controls with no cells and in one case zero activity). The lability of $N_2O_3^{2-}$, the decomposition of which to NO and N_2O is markedly catalyzed by metal ions, is expected to lead to large background levels of gaseous products with whole cells or crude cell-free extracts. We have performed similar experiments with purified *cd*- and Cu-containing nitrite reductases that have been extensively treated to minimize contamination by adventitious metal ions, and still find relatively high background levels of gaseous decomposition products (Hulse, C.; Weeg-Aerssens, E.; Tiedje, J. M.; Averill, B. A., unpublished results).

Even if the data of Garber, Wehrli, and Hollocher[10] are accepted at face value, their interpretation is open to question. Examination of the enzymological literature reveals no general answer to the question of what one should expect when an enzyme is confronted with a putative intermediate that does not normally dissociate from the enzyme. There are, however, several specific cases in which this phenomenon has been examined. For example, oxaloacetate and NADPH are postulated as nondissociable intermediates in the reaction of malic enzyme, yet the conversion

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of oxaloacetate and NADPH to L-malate and $NADP^+$ is catalyzed by the enzyme at only 10% of the V_{max} with NADPH, CO_2, and pyruvate.[20] Similarly, formyl phosphate is an enzyme-bound intermediate in the formyltetrahydrofolate synthetase reaction, yet it is turned over by the enzyme at ca. 3% of the rate of the normal substrates (MgATP, H_4folate, and formate).[21] These results have been explained in terms of a sequential mechanism with a kinetically trapped intermediate (i.e., one with both a slow dissociation *and a slow binding step*).[21] Similar behavior for species V in Scheme V is not unreasonable and would render detection of enzymatic activity difficult with a labile substrate such as $N_2O_3^{2-}$. In the case of formyltetrahydrofolate synthetase, the lability of formyl phosphate prevented detection of catalytic activity with it as a substrate for over 25 years.[22,23]

The other major alternative mechanism for denitrification, proposed over a decade ago, postulates the existence of two enzymes, a nitrite reductase that produces NO as the sole product and a separate NO reductase that reduces two NO molecules to N_2O[24] (cf. Scheme I). The evidence supporting the existence of two enzymes and NO as a free obligatory intermediate is as follows: (i) the observation that purified nitrite reductases produce only NO from NO_2^-, while at least small amounts of NO reductase activity are found in other fractions;[25-28] (ii) denitrifying bacteria and cell-free suspensions produce and consume NO during nitrite reduction;[29-34] (iii) nitrite reductase catalyzes exchange of N between isotopically labeled nitrite and a pool of added NO during reduction of NO_2^- to N_2O;[35,36] and (iv) formation of EPR signals due to ferrous heme–NO complexes upon addition of nitrite to purified nitrite reductase.[37-39]

All of the above evidence, however, can be equally well explained in terms of a sequential mechanism catalyzed by a single enzyme (Scheme V). Even though there is substantial evidence for the existence of two crude fractions in cell-free extracts of denitrifiers, this has not matured into proof, as a purified NO reductase has thus far eluded all investigations.[24,33,40-43] The chemical reactivity of NO makes it reasonable to suggest that at least some conversion of NO to N_2O may be due to secondary or nonphysiological activities of other cellular components, as shown recently by Zumft for ferrous iron–ascorbate mixtures.[44] Whether this is in fact the case, the existence of a separate NO reductase activity is irrelevant to the chemistry associated with the heme *cd* nitrite reductase, which can account for all of the above observations. For example, arguments (ii) and (iii) above can be readily interpreted in terms of the reactivity of the ferrous–nitrosyl intermediate III, as shown in the bottom portion of Scheme V. Studies with synthetic heme nitrosyls[17,18] indicate that, in contrast to ferrous heme–NO complexes, the NO of the one-electron oxidized species is labile (reaction III $\rightleftarrows$ IX), producing the ferric heme and free NO. This reaction would account for the production and consumption of NO by denitrifiers, for the small and relatively constant pool of NO observed during reduction of nitrite,[29,34,36] for the exchange of labeled N between NO_2^- and added NO,[35,36] and for N_2O production,[24] cell growth,[32] active transport,[45] and proton translocation[7] with NO as sole electron acceptor, since the dehydration reaction (II $\rightleftarrows$ III) is known to be reversible.[5,8,46] The traditional explanation for argument (iv) above has been the sequence $NO_2^- \rightarrow$ ferrous heme·NO $\rightarrow$ NO. Since the dissociation of NO from ferrous heme–NO complexes is extraordinarily slow (even slower than CO dissociation[47]), this sequence seems unlikely. Indeed, there is no evidence that the ferrous heme–NO complex forms or decays within the turnover time of the enzyme (i.e., that it is kinetically competent). As indicated in Scheme V, this species (X) can form in a variety of ways and is irrelevant to the catalytic mechanism.

Finally, an alternative sequential mechanism exists that is also consistent with the results reported herein. This involves a reaction of a coordinated nitroxyl anion (Fe–NO^-) with NO_2^- to produce coordinated oxyhyponitrite (Fe–$N_2O_3^{2-}$; species V in Scheme V) directly, and this has as precedent the known reaction of HNO with NO_2^- to produce $HN_2O_3^-$.[48] Recent electrochemical studies by Kadish,[19] Fajer,[49] and Meyer[50] have shown, however, that it is very difficult to reduce ferrous heme–nitrosyl complexes (Fe^{2+}–NO·). Reported reduction potentials for the Fe^{2+}–NO·/Fe^{2+}–NO^- couple with porphyrin and related ligands are in the range of −0.6 to −0.9 V vs SHE[19,49,50] and do not vary greatly with the nature of the macrocyclic ligand (porphyrin vs chlorin vs isobacteriochlorin[49,50]) or the axial ligand.[19,49,50] Since the

Table V. ^{18}O Isotopic Enrichment of Denitrification Products as a Function of Nitrite Concentration for Sonicated Crude Cell-Free Extracts[a]

NO_2^-, mM	N_2O-44	N_2O-46	% equilib
10	20 131 177	465 193	23.4
	20 881 770	621 293	30.5
			av 27.0 ± 3.6
1	21 923 078	1 254 134	59.2
	28 666 146	1 692 224	61.1
			av 60.2 ± 1.1

[a] Numbers are integrator counts for a standard injection volume. Conditions: reductant = 100 mM sodium succinate; medium contained 9 atom % $H_2{}^{18}O$.

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Table VI. Competition between Nitrosation and Denitrification of Crude Extracts Prepared by Sonication[a]

$Na^{15}NO_2$, mM	N_2O-45	N_2O-46	$\frac{N_2O\text{-}45}{N_2O\text{-}46}$
0.05	20 020	164 100	0.122
	21 380	168 200	0.127
			av 0.125 ± 0.002
0.10	18 220	375 750	0.048
	26 080	369 900	0.071
			av 0.060 ± .010
1.0	21 270	405 950	0.052
	18 990	420 780	0.045
			av 0.048 ± 0.003
10.0	82 330	449 140	0.183
	76 840	462 700	0.166
			av 0.175 ± .007

[a] Conditions: as in Table I except for reductant = 100 mM sodium succinate; crude extracts prepared by sonication.

biological reductant is either ascorbate ($E_0' = +60$ mV) or succinate ($E_0' = +30$ mV) and E_0' for the NO_2^-/N_2O couple is +0.77 V, it is difficult to accept the intermediacy of such a strongly reducing species as the coordinated nitroxyl anion in denitrification. (This argument is also relevant to the nitroxyl mechanism proposed earlier by Hollocher[5] and discussed above).

Effect of Enzyme Solubilization on the Fate of the Nitrosyl Intermediate. If, as seems plausible, a common mechanism obtains for at least all heme cd_1 containing nitrite reductases, the finding of Garber and Hollocher[37] that different denitrifiers exhibit varying degrees of ^{18}O exchange into product N_2O suggests that differences in active-site environment may affect the partitioning of the nitrosyl intermediate (III) among the three competing reactions shown in Scheme V: reaction with NO_2^- to give IV and thence N_2O; internal electron transfer to give NO and the ferric heme, IX; or reduction to X. A similar change in active-site environment may also explain the apparent shift from N_2O production in whole cells or cell-free extracts to NO production in purified heme cd_1 nitrite reductase. All that is required is that the relative rates of reactions III → IV and III → IX in Scheme V are reversed in the purified vs membrane-associated enzyme. We have now obtained preliminary data using isotope-labeling studies that suggest that the fate of the nitrosyl intermediate is indeed affected by the extent of solubilization the enzyme.

We have shown previously[13] with whole cells of *P. stutzeri* that the extent of ^{18}O exchange between $H_2{}^{18}O$ and product N_2O is a function of the nitrite concentration. The extent of equilibration ranged from 35% at 90 μM nitrite to 7.9% at 9 mM nitrite. We have now repeated this experiment using cell-free crude extracts prepared by sonication. As shown in Table V, the same trend toward increased ^{18}O equilibration at lower nitrite concentrations is observed, with the sonicated extracts, but all values are 3–4-fold higher than the results with whole cells.

The competition experiments described in Tables I–III above employed cell-free crude extracts prepared by using a French press. For nitrite concentrations ranging from 50 μM to 1 mM and azide concentrations of 50 mM, we found that the fraction of total N_2O formed via denitrification ranged from 56 to 85%. Data obtained under the same conditions for cell-free extracts prepared by sonication are given in Table VI. Even though the general trend is again the same, with an increasing fraction of N_2O due to denitrification with increasing nitrite concentration, the actual figures are very different, with nitrosation accounting for only 5–10% of total N_2O. (Once again the supply of proximal reductant, probably a reduced cytochrome, apparently becomes rate-limiting at high $[NO_2^-]$, as noted previously in Table II.)

These observations, although preliminary in nature, suggest that isotope labeling studies can be used to probe alterations in relative rates of individual steps within the catalytic mechanism as the enzyme is purified. More detailed studies will be required to correlate observed changes in relative rates with the physical state of the enzyme (e.g., lipid content, degree of aggregation) and are in progress. Nontheless, these results suggest that it is not unreasonable to look to perturbations in active-site environment to explain the shift in product from N_2O to NO as the enzyme is purified, rather than invoking the existence of separate NO_2^- and NO reductases.

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Registry No. NO_2^-, 14797-65-0; NO, 10102-43-9; N_2O, 10024-97-2; N_3^-, 14343-69-2; nitrite reductase, 9080-03-9.