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MINIREVIEW

Denitrification: Production and Consumption of Nitric Oxide

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Denitrification has been known for more than a century and is widely recognized as a key process in the biogeochemical nitrogen cycle. It is the major mechanism that converts combined nitrogen, the form available to eukaryotes, to dinitrogen gas, thereby completing the nitrogen cycle. In recent years, denitrification has taken on added importance for the following reasons. First, it is a major source of NO and N_2O, gases that are of focal importance to atmospheric ozone destruction and to global warming. Indeed, N_2O concentrations in the atmosphere have been increasing at 0.2 to 0.3% per year for at least 20 to 30 years (44), and N_2O along with CO_2 and CH_4 are the most important gases thought to be driving climate change. More recently, significant fluxes of NO from soils to the atmosphere have been measured (13), raising questions about the microbial sources of this gas. Second, denitrification is important to waste treatment as a means of both removing excess nitrate and stimulating carbon removal when aeration is difficult. In the latter case, there is increased interest in using nitrate to drive pollutant bioremediation in aquifers (33), because nitrate is more water soluble and mobile in soil than is oxygen. Third, denitrification below the rooting zone has been largely ignored, but recent evidence shows that it is important to an understanding of the carbon, nitrogen, and mineral cycling in the vadose zone, aquifers, and deeper geological formations. Fourth, the discovery that NO is a key chemical signal in a variety of mammalian functions, including the cell killing function of macrophages, neurotransmission, and control of smooth muscle, led to NO being named 1992 Molecule of the Year by *Science* magazine (37). Whether microbial colonizers of mammals play any role in production or consumption of bioactive NO is unknown. Similarly, whether there is any mechanistic insight to be gained by comparing microbial and mammalian NO binding or transformation is just beginning to be explored. These reasons all speak to the importance of gaining a basic understanding of denitrification. This review summarizes recent advances in the physiology, biochemistry, and genetics of the central steps in denitrification, nitrite and nitric oxide reduction.

NITRIC OXIDE AS AN INTERMEDIATE IN DENITRIFICATION

One of the most important recent developments in denitrification has been the elucidation of the pathway from NO_2^- to N_2O. Several models had been proposed for this conversion, but it is now clear that at least in most denitrifiers reduction of NO_2^- occurs in two enzymatic steps, with NO as an obligatory intermediate. Thus, four enzymatic steps are now thought to be required to convert nitrate to dinitrogen gas during denitrification. The current understanding of this pathway and the organization of its enzymes with respect to the cell membrane in gram-negative bacteria are summarized in Fig. 1. The overall organization of denitrification in gram-positive bacteria has not been well studied.

Two main hypotheses have guided denitrification pathway research over the past decade. One hypothesis stated that conversion of NO_2^- to N_2O might be carried out entirely by nitrite reductase via a process in which an enzyme-bound nitrosyl (NO^+) formed by dehydration of NO_2^- underwent nucleophilic attack by a second nitrite to form enzyme-bound N_2O_3 (3). This hypothesis is referred to as the direct pathway, since NO is not an obligatory free intermediate. The other hypothesis describes a sequential pathway involving two enzymes, nitrite reductase and nitric oxide reductase, with NO as a free intermediate ($NO_2^- \rightarrow NO \rightarrow N_2O$). This classical hypothesis is known as the indirect pathway. NO was suggested as an intermediate in 1910, but it was not until Payne's 1973 review that evidence was sufficient to hypothesize its pathway stature (43b). The understanding of the role of NO was preliminary, and further studies raised doubts about whether NO was a free intermediate (3, 20, 56). Only in the past few years has it become clear that NO is an obligate intermediate in respiratory denitrification, at least for most denitrifiers. The key evidence is as follows. (i) Nitric oxide reductases have finally been isolated and characterized (8, 14, 28). (ii) Nitrite reductases have been shown to produce NO as the major product (9, 36, 57). (iii) Nir^- mutants have been prepared and shown to reduce NO to N_2O (59, 60, 68). (iv) Nor^- mutants have been prepared and shown to accumulate NO, making the Nor^- phenotype lethal (6). (v) Measurement of steady state levels under various conditions and trapping studies have shown NO to possess the properties of a kinetically competent intermediate (5, 24–26, 63). (vi) $H_2{}^{18}O$ exchange studies have demonstrated the existence of an electrophilic NO-derived species that exchanges oxygen with $H_2{}^{18}O$ during reduction of NO as well as NO_2^- (62), explaining results previously attributed solely to the nitrite reductase. Key evidence is discussed below.

NO is the major product of dissimilatory reduction of nitrite. Purified nitrite reductases studied so far produce NO as the major product, with N_2O as a minor product (9, 36, 57). Most of the N_2O produced can, however, be abolished by chelators such as EDTA, suggesting chemical conversion of NO to N_2O by trace amounts of Fe contamination (58, 66). Triton X-100 was also shown to inhibit NO reduction in crude extracts of several denitrifiers, and when assayed with nitrite, 80 to 95% of the nitrogen was recovered as NO (46). To quantify the amount of NO diffusible out of the cell during reduction of nitrite or nitrate, extracellular deoxyhemoglobin trapping (24) and gas sparging (63) were used. Most of the

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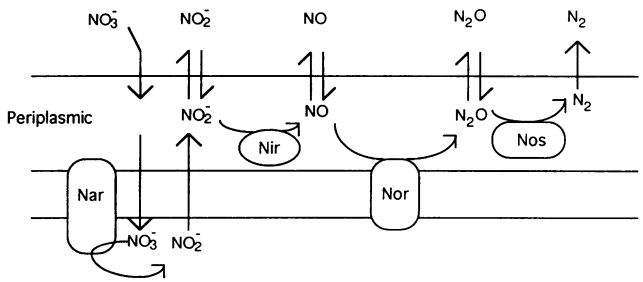

FIG. 1. Denitrification pathway in gram-negative bacteria. Nar, nitrate reductase; Nir, nitrite reductase; Nor, nitric oxide reductase; Nos, nitrous oxide reductase.

nitrogen was again recovered as NO (>70%). These experiments demonstrated that NO can diffuse out of the cell and that most of the N atoms from nitrite or nitrate are found in NO before it is reduced to N_2O, both in vivo and in vitro.

If NO is an intermediate during the reduction of nitrite to nitrous oxide, then exogenous NO would have a nearly completely random combination (scrambling) between the exogenous N and endogenous N to form N_2O during simultaneous reduction of nitrite and NO. This phenomenon was observed in N labelling (^{13}N or ^{15}N) experiments with *Pseudomonas aureofaciens*, *Pseudomonas chlororaphis* (16), and *Paracoccus denitrificans* (25). Early observations (20) that there was a lack of extensive scrambling between ^{14}N and ^{15}N was apparently due to a lack of equilibration between the gas and liquid phases in experiments that used a high density of cells (25).

The most convincing proof that NO is the in vivo product of nitrite reductase comes from the isolation of a Nor^- mutant by Braun and Zumft (6). This mutant lacks the ability to convert NO to N_2O, blocking reduction of nitrite at NO. As expected, the Nor^- mutation is conditionally lethal under anaerobic conditions in the presence of nitrate due to the accumulation of toxic NO. A double mutation in both nitrite reduction and NO reduction rendered the bacteria again viable. Both mutants produce a small amount of NO from nitrate, but its origin is not clear.

One concern with NO as an intermediate is its toxicity, particularly its reactivity with heme and nonheme iron-containing proteins. To address the plausibility of low steady-state levels of NO, it was shown that a low concentration of NO allows NO to be an intermediate without reaching toxic steady-state levels (25, 26). This is accomplished by a very low apparent K_m and a higher V_{max} of the nitric oxide reductase than those of the nitrite reductase. Channeling of NO from nitrite reductase to nitric oxide reductase through protein-protein interactions may be another factor limiting NO toxicity.

Nitrite reduction and nitric oxide reduction are two distinct but related processes. Purified cytochrome cd_1 nitrite reductases (cd_1-dNirs) do not have the ability to convert NO to N_2O (36, 58). In crude extracts, when Cu-type nitrite reductases (Cu-dNirs) were inhibited by the chelator diethyl dithiocarbamate the ability to convert NO to N_2O was not affected (48, 60). Nir^- mutants still can reduce NO to N_2O (59, 60, 68). These results, as well as those mentioned above, indicate that nitrite reduction and NO reduction are two distinct processes. However, this does not imply that these two steps do not influence each other. Tn*5* Nir^- mutants obtained from *Pseudomonas fluorescens* AK-15 with a heme-type dNir (59) and *Pseudomonas* sp strain G-179 with a Cu-dNir (60) showed a significant decrease in the rate of NO reduction. In addition, ^{18}O exchange during NO reduction was greatly reduced in Nir^- mutants of *P. fluorescens*. Mutation in *nirQ*, a putative regulatory gene, results in a loss of both nitrite reduction and nitric oxide reduction in vivo (35). In fact, the amount of NO reductase is substantially reduced in Nir^- mutants isolated from *Pseudomonas stutzeri* JM300, although the rate of NO reduction in vivo is normal (35, 65). Conversely, deletion of the nitric oxide reductase genes (*norCB*) resulted in a lower expression of nitrite reductase (65). This evidence suggests that there is a genetic and/or functional interdependence of these two steps in these organisms. Potential coupling between these two steps in vivo needs to be explored.

Reduction of NO is energy conserving. As an intermediate in denitrification, one important function of NO is to serve as an electron acceptor and conserve energy. Proton translocation unique to NO reduction under denitrifying conditions has been demonstrated in a number of denitrifiers (47). Values obtained were consistent with the expected ratios of 0.5 mol of H^+ per mol of NO for reduction of NO to N_2O in *Paracoccus denitrificans* (9). Antimycin A strongly inhibited NO-dependent proton translocation, further suggesting that a proton electrochemical gradient is generated. Thus, the roles of NO reduction are to detoxify and to serve as an electron acceptor for energy conservation.

NO reductases. The isolation of the nitric oxide reductases took a long time to achieve, which contributed to the uncertainty about the catalyst for this step. Two nitric oxide reductases have now been isolated and characterized (8, 14, 28). Most of the NO reductase activity is found in the membrane fraction (27, 28, 66), requiring suitable detergents for purification of the enzyme. Both enzymes isolated to date are cytochrome complexes containing heme *b*, associated with a 37- or 38-kDa subunit, and heme *c*, associated with a 17- or 18-kDa subunit. An initial report suggesting the absence of chromophores other than lipid material (30) is thus apparently unfounded (14). Both NO reductases studied contain two heme groups per mole, but there are several nonheme iron atoms in both enzymes, and the roles of both forms of iron are as yet unknown.

MECHANISM OF NO REDUCTION

The biological evidence described above clearly shows that, at least in most organisms and under most conditions, NO is a free intermediate in the denitrification pathway. This implies that NO reductase is able to convert two molecules of NO to one of N_2O, although the chemical mechanism remains unclear. Previous studies on the reduction of nitrite by the heme cd_1 nitrite reductase have demonstrated the formation of an electrophilic nitrosyl complex, $E\text{-}NO^+$, by ^{18}O exchange with $H_2{}^{18}O$ and by trapping with nucleophiles (36). More recent studies on the mechanism of NO reduction by crude extracts and whole cells showed similar characteristics: (i) exchange of oxygen with $H_2{}^{18}O$, resulting in production of $N_2{}^{18}O$ (62); (ii) a decrease in ^{18}O exchange with increased electron flux; (iii) trapping of an NO-derived species with nucleophilic compounds; and (iv) ^{18}O exchange with $H_2{}^{18}O$ into the NO pool to give $N^{18}O$ (58). These findings suggest the formation of an electrophilic enzyme-bound nitrosyl species, $E\text{-}NO^+$, during dissimilatory reduction of NO but do not demonstrate whether such a species is an intermediate in N_2O formation or whether it is due to a side reaction that is suppressed by increasing the supply of reductant (62).

The realization that NO, as a denitrification intermediate, can undergo ^{18}O exchange was unexpected and raises questions about previous studies on the mechanism of N=N bond

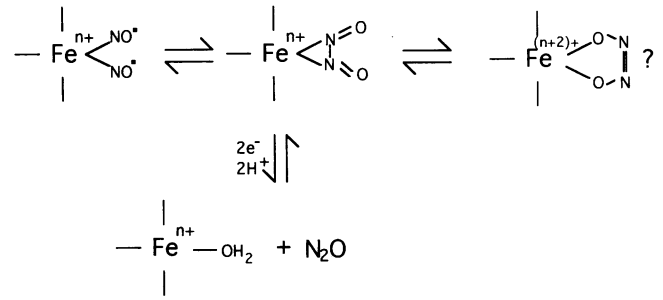

FIG. 2. Possible mechanism for reductive coupling of two NOs to form N_2O at a nonheme Fe site in NO reductase.

formation from nitrite (21, 56). Those studies utilized crude extracts or whole cells, and consequently it is impossible to determine whether the observed ^{18}O exchange and nitrosyl trapping occurred during the reduction of NO_2^- to NO or during the reduction of NO to N_2O. Thus, interpretation of these previous experiments in terms of exclusive formation of N_2O by the nitrite reductase is probably not valid.

Indirect evidence has been presented for the existence of the nitroxyl anion, NO^-, as an intermediate in NO reduction by the NO reductase of *Paracoccus denitrificans* (54). Since it is known that NO^-, once formed, rapidly dimerizes to produce N_2O (52), this proposal suggests that the N=N bond of N_2O is formed by a spontaneous nonenzymatic chemical reaction following a one-electron reduction of NO by NO reductase. Although potentially attractive, this proposal has one major problem. If the active site of NO reductase is a heme iron center (either the heme *c* or heme *b*), then it is not clear how a heme $Fe^{2+}NO^-$ species can be produced on thermodynamic grounds. Available data indicate that the potential for reduction of heme Fe^{2+}-NO^0 species to the Fe^{2+}-NO^- state is in the range of -0.9 V and does not depend greatly on either the nature of the heme or the identity of the ligand *trans* to the NO (16a, 43a). Typical reductants in vitro or in vivo are succinate (+0.03 V) and ascorbate (+0.06 V), which are thermodynamically incapable of producing an intermediate at -0.9 V, especially when the potential for reduction of NO to N_2O is +1.18 V. In contrast, the potential for the heme Fe^{2+}-NO^+/Fe^{2+}-NO^0 couple ranges from ca. +0.39 to -0.02 V, well within the physiologically accessible range. Thus, it is not clear how an NO reductase can generate NO^- coordinated to a heme, which is energetically uphill, during the thermodynamically favorable reduction of NO to N_2O. There is, however, evidence from EPR spectroscopy for formation of an iron nitrosyl complex by the NO reductase (65).

In view of this difficulty with NO^- as an intermediate, we have considered alternative mechanisms. Reductive coupling of two NO molecules to N_2O at a heme center could appear to be ruled out by the difficulty in coordinating two NO molecules on the same side of the heme plane (3). Reductive coupling of NO at nonheme metal centers has ample precedent in organometallic chemistry (32, 42), and it has been shown that NO can be converted to N_2O chemically by Fe^{2+} in assay systems for nitrite and nitric oxide reductases (58, 66). The two NO reductase preparations reported to date contain substantial amounts of tightly bound nonheme iron. Thus, one potentially attractive possibility is reductive coupling of NO at a nonheme iron active site (Fig. 2). In this model, the cytochrome *c* and *b* centers would act as electron transfer sites for donation of electrons to the nonheme active site. If, as seems likely, the heme centers can also bind NO (as do virtually all cytochromes known), then it is possible that the observed $H_2{}^{18}O$ exchange results from a small population of the oxidized heme Fe^{2+}-NO^+ center in equilibrium with the Fe^{2+}-NO^0 species but that this reaction has nothing to do with the active site chemistry. Further work to distinguish among these possibilities as well as those previously suggested (62) is clearly necessary.

ENZYMOLOGY OF NITRITE REDUCTION

Cu-containing nitrite reductases. Denitrifiers with Cu-dNirs constitute one-third of the numerically dominant isolates from soil (11, 18). Most of the Cu-dNirs cross-react with polyclonal antibodies raised against the Cu-dNirs, suggesting that most Cu-dNirs share substantial similarity (11, 41).

The nitrite reductase from *Achromobacter cycloclastes* is the one best studied (15, 23). The enzyme is a trimer with a molecular mass of 36 kDa per monomer and two copper atoms per monomer. The amino acid sequence (15) and 0.23-nm X-ray stucture (23) reveal that the two copper atoms in the monomer constitute one type 1 ("blue") copper site and one putative type 2 ("non-blue") copper site. The type 1 copper is bound by residues within the monomer, but the type 2 copper is bound by residues from each of two monomers of the trimer. It had been suggested that the active site of Cu-dNirs is the type 1 copper, since only type 1 copper was detected in the Cu-dNirs isolated from *P. aureofaciens* (67) and *Alcaligenes xylosoxidans* (40) and the type 1 site was found to be reduced upon the addition of NO (53). The following evidence, however, strongly suggests that the type 2 copper center constitutes the active site of the enzyme. (i) Nitrite binds to the type 2 copper site, not to the type 1 Cu (23). (ii) Type 2 Cu can be removed from the enzyme, resulting in essentially no activity. The reconstituted enzyme shows a linear correlation between the type 2 Cu content and activity (38). (iii) Ascorbate oxidase has a type 2 Cu in its active site, and its location in the protein is very similar to that in the Cu-dNir from *Achromobacter cycloclastes* (23). (iv) The amino acid sequence of the Cu-dNir from *P. aureofaciens* (one of two strains in which type 2 Cu was not detected in purified protein) reveals the presence of a type 2 copper-binding site (22). Type 2 copper appears to be relatively weakly held by its ligands and thus may be lost easily from the enzyme during purification. The role of type 1 Cu seems to be to accept electrons from the physiological electron donor and pass them to the type 2 copper active site, similar to the postulated relationship between the heme *c* and heme d_1 sites in the cytochrome cd_1-dNir.

Cytochrome cd_1dNir. Cytochrome cd_1-dNirs have been found in most (ca. two-thirds) of the denitrifiers studied but in fewer genera than contain the Cu-dNirs (11). These enzymes consist of two identical subunits with molecular masses of 60 kDa, each containing one heme *c* prosthetic group covalently linked to the polypeptide chain and one heme d_1 moiety noncovalently associated with the protein (29, 57). Antibodies raised against the cd_1-dNir from *Pseudomonas aeruginosa* cross-react strongly with many other cd_1-dNirs (11). Heme *c* binding ligands (34, 49, 57) are located near the N terminus of the protein, but the heme d_1 binding domain remains to be defined. Heme d_1 is unique to denitrifiers that contain the heme-type nitrite reductases (10). An apoprotein lacking the heme d_1 could be reconstituted with synthetic heme d_1 (57). All of these cd_1-dNir gene sequences reveal the presence of a signal peptide, in agreement with the location of these enzymes in the periplasm (34, 49).

GENETICS OF NITRITE REDUCTION

Characterization of genes involved. An operon containing the cd_1-dNir structural gene (*nirS*) has been studied in *P. stutzeri* (34, 50), *P. aeruginosa* (49), and *P. fluorescens* (59). The first gene in this operon is the nitrite reductase structural gene, *nirS*. Immediately downstream are *nirM*, encoding cytochrome c_{551} (2, 43), and *nirC* (ORF5), encoding an unknown heme protein in *P. aeruginosa* (2) and *P. fluorescens* (69). In *P. stutzeri*, *nirT*, encoding an unknown tetraheme protein, and *nirB*, encoding cytochrome c_{552}, are inserted between *nirS* and *nirM* (34). Apart from those in the *nir* operon, a gene(s) responsible for heme d_1 synthesis (*nirD*) has been identified (68). Cosmid mapping and Southern hybridization revealed a close linkage of the genes involved in nitrite, nitric oxide, and nitrous oxide reduction (7).

Much less is known about the genes involved in nitrite reduction in organisms that contain Cu-dNirs. The structural gene for Cu-dNir (*nirU*) has been isolated from *Pseudomonas* sp. strain G-179 (61) and *P. aureofaciens* (22). The deduced amino acid sequences of both proteins appear homologous to that found for the protein from *Achromobacter cycloclastes*. When the *nirU* gene from *Pseudomonas* sp. strain G-179 was used as a probe, it hybridized to the DNA of most of the Cu-dNir-containing denitrifiers tested but not to the DNA of organisms containing a cd_1-dNir (except for *P. stutzeri* JM300). Thus, the structural gene sequence must be similar in many of the organisms that contain Cu-dNirs.

Regulation of gene expression. Denitrification occurs under oxygen-limiting conditions (<10 μM O_2), except in rare but interesting organisms such as *Thiosphaera pantotropha* (4) (now reclassified as *Paracoccus denitrificans* [39]). The FNR protein is essential for the expression of genes involved in fumarate and nitrate reduction under anaerobic conditions in *Escherichia coli* and other enteric bacteria (51). The conserved symmetrical sequence, $TTGATN_4ATCAA$ (FNR box), is located upstream of the FNR-dependent genes and operons. It has been shown that *anr* (for anaerobic regulation of arginine deiminase and nitrate reduction) encodes a protein that exhibits structural and functional similarity to the FNR protein from *E. coli* (45, 64). ANR^- strains lack the ability to utilize arginine and nitrate under anaerobic conditions. ANR acts on the consensus FNR box to regulate gene expression for the arginine deiminase pathway under anaerobic conditions in *P. aeruginosa* (17, 19). Putative FNR boxes have been found in the promoter regions of many genes involved in denitrification (12, 31, 35, 50, 55, 61). We have recently obtained an ANR^- deletion mutant of *P. aeruginosa* (PAO6261) from D. Hass and found that this mutant failed to grow on nitrate, nitrite, and nitrous oxide under anaerobic conditions, whereas the wild-type strain grew normally on these substrates. Furthermore, crude extracts from the mutant induced under anaerobic conditions had little nitrite reductase and nitric oxide reductase activities, whereas extracts from the wild type had a much higher level of both activities. All of this evidence suggests that an ANR plays a global role in regulation of denitrification under anaerobic conditions in *P. aeruginosa*.

Environmental and physiological studies have shown that substrates for the denitrification pathway, such as nitrate, nitrite, and N_2O, are required for the full expression of enzymatic activities. This finding has been supported recently by studies at the gene level. It was found that cells grown on arginine anaerobically in the absence of nitrate had a lower level of expression of the *nir* operon than cells grown under anaerobic denitrifying conditions with nitrate as the electron acceptor (1). This experiment suggests that the N oxide substrates activate the transcription of the genes involved in nitrite reduction.

CONCLUSIONS

The major recent advance has been to establish that nitric oxide is an intermediate in the denitrification pathway. NO reduction appears to involve a nitrosyl intermediate ($E\text{-}NO^+$), possibly making this step mechanistically similar to NO_2^- reduction. The Cu-dNir is now better understood. Both type 1 Cu and type 2 Cu are required for activity. The steps of nitrite and nitric oxide reduction appear to be linked genetically and functionally, since mutations in one step affect the enzymatic activity of the other. Furthermore, a low level of intermediate, NO, is generated during denitrification, consistent with the channeling mechanism of these two steps. Regulation of denitrification by oxygen may be under the control of an FNR-like protein.

Several issues about nitrite and nitric oxide reduction need to be resolved. (i) The mechanism of NO reduction is poorly understood, which is important since this is the step in which the N=N bond is formed. (ii) Since NO fluxes from ecosystems are of global atmospheric significance, it is particularly important to gain an understanding of how nitrite reductases and nitric oxide reductases are organized and function as a unit, because this is likely to be critical to the amount of NO lost from the cell, and hence from ecosystems. (iii) Very little information is available on the regulation of denitrification, yet this is the key factor that determines when and where denitrification occurs. The model of FNR and substrate regulation in enteric bacteria seems to be an appropriate starting point.

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ADDENDUM

During review of this article, a minireview by Zumft appeared on the role of nitric oxide in bacteria (65). The reader is referred to this paper for a more lengthy treatment of some of the subjects mentioned above.

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