Hypothesis

THE CHEMICAL MECHANISM OF MICROBIAL DENITRIFICATION

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1. Introduction

Denitrification is the dissimilatory reduction of nitrate to volatile products (nitrous oxide or dinitrogen) via nitrite. It is performed by a variety of bacteria that are widely distributed in soils and sediments and that utilize oxidized nitrogen compounds in the absence of oxygen as a terminal electron acceptor. This process is of profound importance for a number of reasons:

(i) It is responsible for major losses of the nutrient most limiting to plant productivity; the best recent estimates suggest that ~30% of fertilized nitrogen is lost via denitrification [1];

(ii) One product of denitrification is nitrous oxide, which if increased due to human activities such as fertilization, could lead to destruction of the ozone layer and to increased heating of the planet;

(iii) Denitrification processes are seeing increased commercial use in waste water treatment plants, where they are used to reduce the available nitrogen concentration of sewage before discharge into streams;

(iv) A key intermediate in the process is nitrite ion, which is known to react readily with secondary amines to form carcinogenic nitrosamines.

In view of the importance of denitrification, elucidation of the details of the process have been the object of research by several groups. This work has resulted in the identification of NO_2^- and N_2O as free obligatory intermediates [2–4]:

$$NO_3^- \rightarrow NO_2^- \rightarrow ? \rightarrow N_2O \rightarrow N_2 \qquad (1)$$

The first step is catalyzed by a well-characterized enzyme, nitrate reductase, and involves a simple 2-electron reduction of nitrate. The enzyme responsible for the third step, again a 2-electron reduction of nitrous oxide to nitrogen, has been isolated [5]; a variety of experiments, however, had earlier suggested its existence [2]. In contrast, the details of the process by which 2 nitrite ions are reduced to form nitrous oxide (a 4-electron reduction) are still the object of controversy. Classical schemes hypothesized that hyponitrous acid ($H_2N_2O_2$) was a likely intermediate [6], but there is now evidence which eliminates this as a free intermediate [7]. The chief point of controversy is whether NO_2^- is reduced directly to N_2O, or whether NO is formed as an intermediate that is subsequently reduced to N_2O [3]. In particular, a variety of evidence has been adduced in favor of the latter, including:

(i) Direct observation of low levels of NO during NO_2^- turnover [8–10];

(ii) The fact that many purified nitrite reductases also reduce NO [11–13];

(iii) The enzyme-catalyzed exchange of nitrogen between isotopically labelled NO_2^- and a pool of added NO during reduction of NO_2^- to N_2O [10,14];

(iv) Observation of EPR signals due to ferrous heme–NO complexes in purified heme-containing nitrite reductases [15–19].

In contrast, in vivo isotopic exchange studies on *Pseudomonas aeruginosa* showed no evidence for the existence of a pool of free NO [4], consistent with its known cytotoxicity and reactivity to a variety of agents.

Since the key feature that distinguishes denitrification from the assimilatory 6-electron reduction of nitrite to ammonia is the formation of a nitrogen–nitrogen bond in the former, we have concentrated on this aspect of the process. Here, we describe a plausible mechanism for conversion of nitrite to nitrous oxide, and show how this is consistent with both the known inorganic chemistry of related systems and available data on relevant biological systems.

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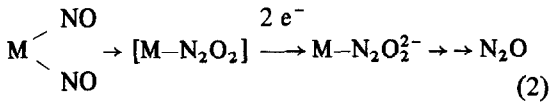
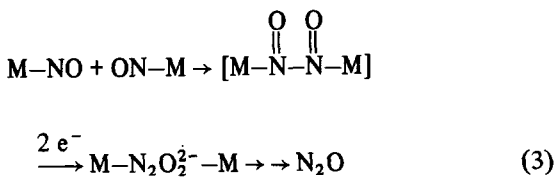
2. Discussion

We have considered 3 possible chemical routes to an N–N-bonded intermediate that could, by subsequent reduction and protonation/dehydration steps, produce N_2O. The first of these (reaction (2)) involves coupling 2 NO radicals at a metal center to form coordinated N_2O_2 as a transient species that would be rapidly reduced to hyponitrite ($N_2O_2^{2-}$). This route would be



(2)

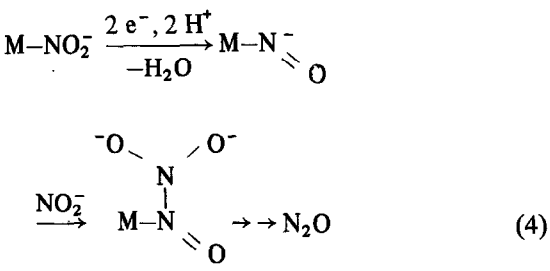
consistent with prior reduction of NO_2^- to free NO, but seems unlikely on chemical grounds:
(i) Known examples of transition metal catalyzed reduction of NO to N_2O (with, e.g., CO as reductant) appear to proceed via coupling of adjacent NO moieties in a *cis*-dinitrosyl complex to either coordinated N_2O_2 or *cis*-hyponitrite [20–23];
(ii) The majority of known dissimilatory nitrite reductases are heme proteins, and heme complexes of first row transition metals such as iron cannot bind 2 molecules such as NO on the same side of the porphyrin plane. The *trans* configuration of a *bis*(nitrosyl)-heme complex [24] would be most unlikely to undergo dimerization. An alternative in which the two NO molecules are coordinated to different metals (reaction (3)) also seems improbable.



(3)

Known examples of such reactions in simple transition metal systems generally involve disproportionation of NO to N_2O and coordinated NO_2^- [20,25], rather than simple reduction as is the case in denitrification. Moreover, the EPR spectra observed for both the ferric [17] and ferrous–NO [15–18] forms of nitrite reductase are typical of those of the analogous forms of simple heme proteins [18,26] and model complexes [18,26,27], with no evidence for magnetic interactions. This suggests that the hemes in these enzymes are relatively well isolated; it is, therefore, unlikely that the bulky heme groups could approach one another closely enough to induce dimerization of bound NOs without substantial reorganization of the protein structure.

The second route considered involves nucleophilic attack of coordinated NO^- on nitrite ion to yield oxyhyponitrite directly:

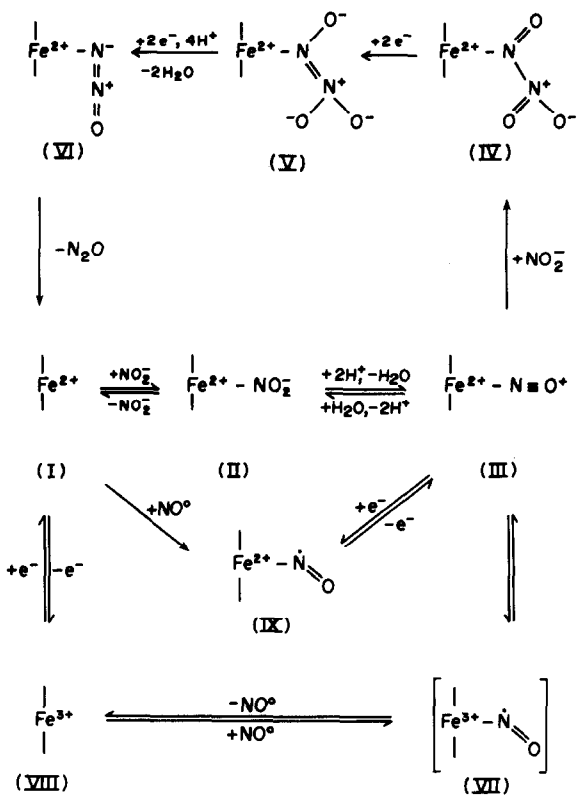


(4)

Again, this seems unlikely on chemical grounds:
(i) Although examples of metal complexes containing coordinated NO^- are known [28], the nitrogen atom of such species apparently has very little nucleophilic character [20,28], inasmuch as it reacts only with powerful electrophiles such as HCl [29], HBr [30], or benzyl bromide [31];
(ii) The nitrite ion at physiological pH has essentially no tendency to act as an electrophile, but instead is a rather good nucleophile [32].

The third route we have considered is shown in detail in the top portion of scheme (1). It is drawn for an enzyme utilizing a ferrous heme prosthetic group, since most nitrite reductases are heme enzymes; an analogous scheme can be drawn for the copper-containing enzymes [33–35]. The scheme involves initial binding of nitrite (II) to an axial position of a ferrous porphyrin (I), followed by protonation and dehydration to the ferrous–nitrosyl complex (III). We propose that complex III can either lose NO, forming the ferric porphyrin (VIII) and providing a facile route for exchange of isotope between NO and NO_2^-, or undergo attack by free nitrite to form N_2O_3 coordinated to the iron (IV). Two-electron reduction of IV will produce coordinated oxyhyponitrite (V), a known species. Further reduction by 2 electrons, protonation, and dehydration will yield a species containing coordinated N_2O (VI), loss of which regenerates the starting ferrous porphyrin (I).

The lower portion of the scheme describes how the evidence previously interpreted in favor of NO as an intermediate can be accounted for. The observed



exchange of isotopes between nitrite and free NO is easily understood, inasmuch as ferrous–nitrosyl complexes such as (III) are known to be relatively unstable, decomposing readily to the ferric porphyrin (VIII) and free NO [24], presumably via internal electron transfer (III → VII). A further point is that the ferrous–NO complex (IX), which has been observed by EPR spectroscopy in several nitrite reductase systems [15–19], has never been shown to be directly involved in either reduction of nitrite to N_2O or in isotope exchange between nitrite and NO, since there is no evidence that it forms or decays within the turnover time of the enzyme. Rather, it is a relatively stable species [26,27] that can be formed via at least 2 routes (see scheme), but it is essentially irrelevant to catalysis.

Several points should be made regarding the chemistry shown in the scheme:

(i) Dehydration of coordinated NO_2^- to form NO^+ is a well-known reaction [36], for example:

$$[Fe(CN)_5(NO_2)]^{4-} \underset{OH^-}{\overset{H^+}{\rightleftharpoons}} [Fe(CN)_5NO]^{2-} \qquad (5)$$

In contrast, although in aqueous solution HNO_2 is known to be in equilibrium with NO^+ [32], the fact that this reaction is important only in strongly acidic solutions makes it of questionable physiological relevance. However, it has been proposed [37] as the first step in nitrite reduction.

(ii) Nucleophilic attack on coordinated NO has ample precedent [38], particularly when the bound NO has substantial positive character and a high N–O stretching frequency (e.g., reaction (5)). This reaction has been demonstrated to occur with nitrosyls of Fe, Ru, and Os for a variety of nitrogenous nucleophiles [38], but not to our knowledge for nitrite. It should be noted that the ferrous porphyrin–nitrosyl complexes appear to be especially powerful nitrosylating agents [24].

(iii) Reduction by 2 electrons of the product of attack by NO_2^- and NO (coordinated N_2O_3) produces a known chemical species, oxyhyponitrite [39], that can be reduced and dehydrated to yield N_2O via hyponitrite ($N_2O_2^{2-}$).

(iv) Recent ^{15}N NMR studies [40] on oxyhyponitrite have shown that the most basic site ($pK_a = 9.7$) is the nitrogen atom shown as coordinated to iron in V. Although there is no strict correlation between basicity and tendency to form metal complexes, this observation together with the known affinity of Fe(II) for unsaturated nitrogenous ligands (rather than oxygen) suggests that complex V is not unreasonable.

In short, all of the intermediate steps involve known chemical species, and each reaction has sound chemical precedent.

The scheme also offers an explanation for the sometimes conflicting literature data on denitrification as well.

(i) Reduction of nitrite is proposed to occur via 2-electron steps. This is consistent with all other known biological oxidation–reduction reactions coupled to phosphorylation, which proceed in 2-electron (or a multiple thereof) steps. Nitrite reduction to nitrous oxide has been shown to be coupled to oxidative phosphorylation [41] and the growth yields/electron [42] and H^+ transported/electron [43] are the same as for each of the other steps in the denitrification sequence ($NO_3^- \rightarrow NO_2^-$ and $N_2O \rightarrow N_2$). Conservation of 2-electron steps also avoids the generation of high-energy, radical intermediates, including the toxic NO.

(ii) Depending on the relative rate constants for III → IV and III → VIII (and also on the relative flux

of electrons and nitrite into the system), one would expect to get varying amounts of a paramagnetic ferrous–NO complex (IX) during turnover of nitrite reductase, as has been observed by EPR spectroscopy. (Note, however, that a combination of nitrite and a reductant such as dithionite generates ferrous–NO complexes of many heme proteins via a non-enzymatic process.) The ferrous–nitrosyl complex (III) offers a convenient pathway for exchange of isotopes between NO (gas) and NO_2^-, inasmuch as dissociation of NO is known to be facile [24]. This side equilibrium may be responsible for the detection of small amounts of NO during turnover [9], and for the fact that most dissimilatory nitrite reductases have some nitric oxide reductase activity as well. Note that the reversibility of the reactions between species VIII and I in the scheme can result in formation of NO_2^- from NO, accounting for the recent report of equilibration of isotopes between pools of NO and NO_2^- by the enzyme [10]. This would also account for formation of N_2O with NO as the only substrate. Observations that have been interpreted as suggesting the presence of two separate pathways for nitrite reduction in various organisms, in particular, the enzyme catalyzed incorporation of nitrogen from labelled NO_2^- into a pool of NO during turnover [10], can be explained equally well in terms of the mechanism proposed here.

(iii) One would expect that the relative rates of the various steps shown would be quite sensitive to minor changes in the environment of the enzyme. Available evidence suggests that, as expected for a respiratory enzyme, nitrite reductase activity in vivo is membrane-associated [44,45]. If solubilization of the enzyme were to cause a significant increase in the rate of release of NO from III, this would explain the discrepancy between the in vivo results [4], showing no evidence for NO as an intermediate, and the results with purified nitrite reductase [8], showing evolution and consumption of free NO, both with the same organism, *Pseudomonas aeruginosa*. Any modification of the enzyme that would affect the binding site for a second nitrite ion and its subsequent attack on coordinated NO^+ would convert the enzyme into one which merely evolves NO, possibly explaining some of the claims for a separate $NO_2^- \rightarrow NO$ step.

More important, perhaps, than explaining old data, the pathway presented here should provide a focus for further experimentation and suggests several new lines of attack on the mechanism of denitrification. For example, complex III should be partitioned among 3 possible reaction paths:

(i) A first-order decomposition to the ferric heme (VIII) with release of NO;
(ii) Reduction to the stable ferrous heme–NO complex (IX);
(iii) Reaction with nitrite to form IV and thence to N_2O.

Since only the first of these can result in exchange of isotopes between NO_2^- and NO, this suggests that the extent of isotopic exchange should be sensitive to the relative concentrations of reductant and nitrite. Similarly, if the enzyme has a site designed to promote the reaction of NO^+ and NO_2^-, and hence to stabilize coordinated N_2O_3, it is not unreasonable to suppose that oxyhyponitrite ($N_2O_3^{2-}$) should be a substrate of the enzyme, yielding N_2O upon reduction by 2 electrons. Experiments to test the validity of our proposed scheme by examining these and other points are in progress.

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