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Review

Nitrous oxide reductase

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ABSTRACT

Nitrous oxide is a potent greenhouse gas, whose atmospheric concentration has been increasing since the introduction of the Haber Bosch process led to the widespread use of nitrogenous fertilizers. One of the pathways to its destruction is reduction to molecular nitrogen by the enzyme nitrous oxide reductase found in denitrifying bacteria. This enzyme catalyzes the last step of the denitrification pathway. It has two copper centers, a binuclear CuA center, similar to the one found in cytochrome *c* oxidase, and the CuZ center, a unique tetranuclear copper center now known to possess either one or two sulfide bridges. Nitrous oxide reductase has been isolated in different forms, depending on the oxidation state and molecular forms of its Cu centers. Recently, the structure of a purple form, which has both centers in the oxidized state, revealed that the CuZ center has the form $[Cu_4S_2]$. This review summarizes the biogenesis and regulation of nitrous oxide reductase, and the spectroscopic and kinetic properties of nitrous oxide reductase. The proposed activation and catalytic mechanism, as well as, electron transfer pathways are discussed in the light of the various structures of the CuZ center.

Abbreviations: Ac, *Achromobacter*; B, *Bacillus*; CD, circular dichroism; Dnr, dissimilative nitrate respiration regulator; EPR, electron paramagnetic resonance; Fnr, fumarate and nitrate reductase regulator; Ma, *Marinobacter*; MCD, magnetic circular dichroism; N_2OR, nitrous oxide reductase; *nir*, nitrite reductase; *nar*, nitrate reductase; Nnr, nitrite and nitric oxide reductases regulator; *nor*, nitric oxide reductase; *nos*, nitrous oxide reductase; Pa., *Paracoccus*; Ps., *Pseudomonas*; RR, resonance Raman; Sec, secretory pathway; Tat, twin-arginine translocation; Wo., *Wolinella*.

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1. The environmental relevance of nitrous oxide and the denitrification pathway

Nitrous oxide is a potent greenhouse gas that has a global warming potential more than 300 times higher than that of carbon dioxide [1]. Even though it comprises only 0.03% of the total greenhouse gases (carbon dioxide, methane and nitrous oxide) emissions it contributes substantially to global warming [1]. Moreover, in the last 100 years, the atmospheric concentration of nitrous oxide relative to air has increased almost 20%, and is currently 322 ppb [2,3]; on average there has been a yearly increase of 0.25% in its concentration [4].

Nitrous oxide has a long atmospheric lifetime, taking approximately 120 years to decrease the initial emission by 63% [4,5]. Removal from the stratosphere occurs through photolysis [5] followed by reaction with excited oxygen atoms, reactions that are also responsible for the depletion of the ozone layer [2,6].

The emission of nitrous oxide into the atmosphere has several sources, with the majority being produced by microbial metabolism of nitrogen compounds in soils and oceans, and with the human activities also contributing significantly for its emission [3,4]. From these activities, agriculture is the main contributor due to the increased use of fertilizers and application of live-stock manure in crop lands and pasture [5]. Other human activities that have a major impact in nitrous oxide emissions are fuel combustion and, to a lesser extend or not fully accounted for, human sewage, waste water treatment plants, and burning of biomass and biofuels [5].

The emission of nitrous oxide from microbial processes is derived mainly from two metabolic pathways belonging to the nitrogen biogeochemical cycle: nitrification, which is an oxic process and denitrification, an anoxic or near anoxic process [7–9] (Fig. 1). These pathways are carried out mainly by proteobacteria, though methanotrophic bacteria and fungi also contribute to the release of nitrous oxide using metabolic pathways still only poorly explored [10–15].

One of the bacterial pathways, denitrification, that leads to the release of nitrous oxide to the atmosphere involves the reduction of the nitric oxide intermediate [15]. In the case of nitrification (an autotrophic pathway involved in the aerobic oxidation of ammonia to nitrate, Fig. 1) nitric oxide is produced by the aerobic oxidation of hydroxylamine [17,18], which is favored under low nitrite and high ammonia concentrations [19]. Also, under oxygen-limiting conditions or under high nitrite concentrations, ammonium oxidizing bacteria can reduce nitrite to nitrous oxide in combination with ammonia oxidation (nitrifier denitrification, Fig. 1) [20–22] (Fig. 1).

However, under anaerobic conditions, denitrifying organisms perform the reduction of inorganic nitrate or nitrite in sequential steps that involve the abstraction of an oxygen atom at each step, with the production of gaseous molecules at intermediate stages: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. Each reaction is catalyzed in a concerted way by a different enzyme: nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase (N_2OR), either located in the internal membrane or in the periplasm of several proteobacteria (alpha, beta, gamma and epsilon division). In archaea a similar pathway has been proposed, though the four reductases are membrane bound and dependent on menaquinol as the electron donor [23]. The denitrification pathway allows the bacteria to survive under anaerobic conditions, since there is the generation of an electrochemical gradient across the cytoplasmic membrane, and thus leads to energy conservation [24].

Recently, it has been shown that Gram-positive bacteria, such as some *Bacillus* (*B.*) strains, are also denitrifying organisms able to reduce nitrate or nitrite with the production of nitrous oxide or molecular nitrogen [25,26]. However, the molecular systems involved in this reduction have only been partially identified at the protein level for *B. azotoformans* [27]. The reasons for the denitrification pathways being overlooked in these organisms has been attributed to the low number of completely sequenced Gram-positive genomes identified as denitrifying organisms, the low DNA sequence homology of the putative proteins with the Gram-negative counterparts and also the possibility of new unknown genes being involved in this pathway [28]. Surprisingly, in the case of N_2OR, a gene that shares around 35% sequence homology with those found in proteobacteria, coding for N_2OR has been identified [29] (see Section 2.2).

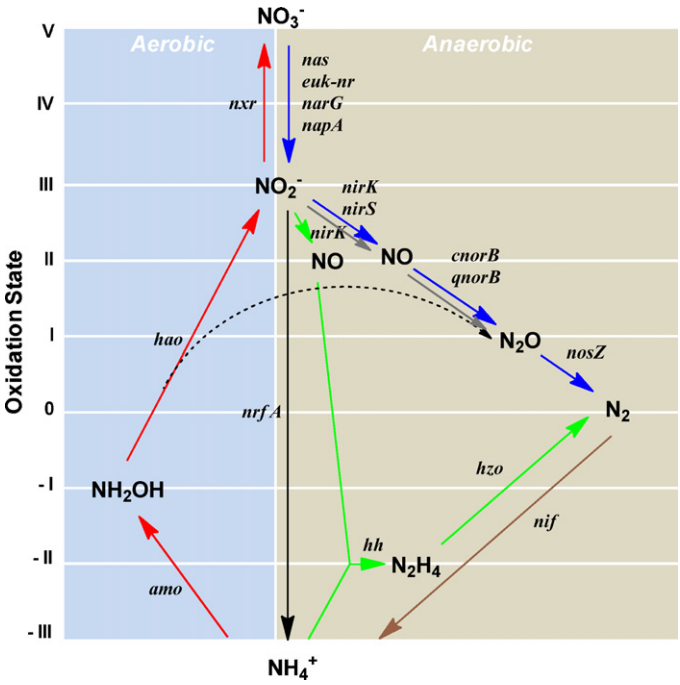

Fig. 1. Biological pathways involved in the nitrogen cycle: denitrification (blue), nitrification (red), anaerobic ammonium oxidation (Anammox) (green), nitrogen fixation (brown) and dissimilatory nitrite reduction to ammonium (DNRA) (black), and dissimilatory nitrite reduction pathway of aerobic nitrifier denitrification (gray). The gene encoding the catalytic subunit of the enzyme(s) that catalyses each reaction is indicated on top of each arrow: different nitrate reductases (*nas*—nitrate reductase cytoplasmic prokaryotic-assimilatory, *euk-nr*—nitrate reductase cytoplasmic eukaryotic-assimilatory, *narG*—nitrate reductase membrane bound dissimilatory, *napA*—periplasmic nitrate reductase dissimilatory), nitrite reductases (*nirK*, *nirS*), nitric oxide reductase (*cnorB*, *qnorB*), N_2OR (*nosZ*), nitrogenase (*nif*), hydrazine hydrolase (*hh*), hydrazine oxidoreductase (*hzo*), dissimilatory nitrite reductase (*nrf*), ammonium monooxygenase (*amo*), hydroxylamine oxidoreductase (*hao*) and nitrite oxidoreductase (*nxr*).
Figure adapted from [16].

On the other hand, in Gram-negative bacteria, these molecular systems have been extensively studied for many years. The different genes that code for the enzymes and electron shuttle proteins involved in denitrification have been identified and those required for its assembly are for the most part known (Table 1). However, their catalytic mechanism, metal cluster assembly, and regulatory systems are not yet completely unraveled.

Recently, one more step toward a better understanding of the denitrification pathway has been achieved with the three-dimensional structure determination of nitric oxide reductase from *Pseudomonas* (*Ps.*) *aeruginosa* (a Gram-negative bacteria), a *c*-type nitric oxide reductase (*c*Nor) [30] and a quinol-dependent nitric oxide reductase from *Geobacillus stearothermophilus* (a Gram-positive bacteria) [31].

Each step of the denitrification pathway can be catalyzed by more than one type of enzyme (Table 1). In the case of nitrite reductase, two different enzymes have been isolated, both with two redox centers in two separate structural domains, one being the electron transfer center and the other the catalytic center, but