Role of nitrogen oxides in the metabolism of ammonia-oxidizing bacteria

M.J. Kampschreur*[1], N.C.G. Tan†, C. Picioreanu*, M.S.M. Jetten†, I. Schmidt‡ and M.C.M. van Loosdrecht*

*Department of Biotechnology, Delft University of Technology, The Netherlands, †Department of Microbiology, IWWR, Radboud University Nijmegen, The Netherlands, and ‡Department of Microbiology, University of Bayreuth, Germany

Abstract

Ammonia-oxidizing bacteria (AOB) can use oxygen and nitrite as electron acceptors. Nitrite reduction by *Nitrosomonas* is observed under three conditions: (i) hydrogen-dependent denitrification, (ii) anoxic ammonia oxidation with nitrogen dioxide (NO_2) and (iii) NO_x-induced aerobic ammonia oxidation. NO_x molecules play an important role in the conversion of ammonia and nitrite by AOB. Absence of nitric oxide (NO), which is generally detectable during ammonia oxidation, severely impairs ammonia oxidation by AOB. The lag phase of recovery of aerobic ammonia oxidation was significantly reduced by NO_2 addition. Acetylene inhibition tests showed that NO_2-dependent and oxygen-dependent ammonia oxidation can be distinguished. Addition of NO_x increased specific activity of ammonia oxidation, growth rate and denitrification capacity. Together, these findings resulted in a hypothetical model on the role of NO_x in ammonia oxidation: the NO_x cycle.

Introduction

Ammonia-oxidizing bacteria (AOB) have been described as obligatory aerobic chemolithoautotrophic organisms for over 100 years. The only known pathway to gain energy was assumed to be aerobic oxidation of ammonia to nitrite. However, the appearance of aerobic nitrifiers in anoxic environments [1,2] indicated that AOB are more versatile than previously assumed. It was discovered that *Nitrosomonas eutropha* could also use nitrite as electron acceptor, which was observed under three conditions: (i) hydrogen-dependent denitrification, (ii) anoxic ammonia oxidation with NO_2 [3] and (iii) NO_x-induced aerobic ammonia oxidation. *Nitrosospira* spp. are also capable of nitrite reduction, suggesting that denitrification could be a universal trait in the β-proteobacterial AOB [4,5].

The pathways of aerobic and anoxic, NO_2-dependent, ammonia oxidation are summarized in Scheme 1. The main products of ammonia oxidation are nitrite under oxic conditions and dinitrogen gas (N_2), nitrite and NO under anoxic conditions. Both aerobic and anoxic ammonia oxidation to hydroxylamine is initiated by the enzyme AMO (ammonia mono-oxygenase). Ammonia, oxygen and NO_2 are the substrates for this enzyme. Comparison of experiments at 25 and 4°C indicated that N_2O_4, and not NO_2, is the actual molecule that is used [6]. The free energy change ($\Delta G^{\circ\prime}$) indicated that anoxic ammonia oxidation [$-120\ kJ \cdot mol^{-1}$; eqn (2)] is slightly more exergonic than oxic ammonia oxidation [$-120\ kJ \cdot mol^{-1}$; eqn (1)]:

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad (1)$$

$$NH_3 + N_2O_4 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O + 2NO \quad (2)$$

The hydroxylamine is in both cases further oxidized to nitrite by hydroxylamine oxidoreductase:

$$NH_2OH + H_2O \rightarrow HNO_2 + 4H^+ + 4e^- \quad (3)$$

Under anoxic conditions, the nitrite produced is partly used as electron acceptor, leading to the formation of N_2 [3]:

$$HNO_2 + 3H^+ + 3e^- \rightarrow 0.5N_2 + 2H_2O \quad (4)$$

Under oxic conditions, with low NO_x concentrations present, *N. eutropha* also uses nitrite as an additional electron acceptor, producing N_2 and traces of nitrous oxide (N_2O) [7].

In the absence of both dissolved oxygen and NO_2, *N. eutropha* and *N. europaea* are capable of nitrite reduction with molecular hydrogen or acetate as electron donor [8]. The enzymes involved in the denitrification pathway in *N. europaea* seem to be regulated by a new type of protein [9].

Significance of NO_x in aerobic ammonia oxidation

NO_x plays an important role in the aerobic metabolism of nitrifying micro-organisms also when not added artificially. Release of NO during ammonia oxidation is well known, but was previously interpreted as formation of a by-product of nitrification, denitrification or chemodenitrification without

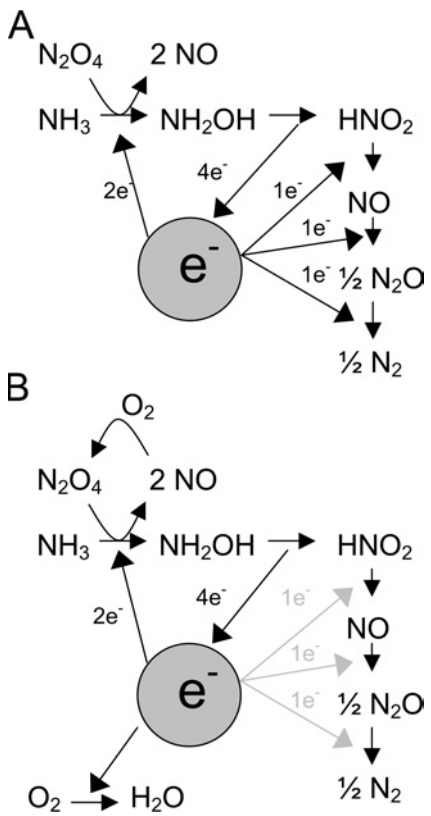
Key words: ammonia oxidation, nitric oxide (NO), nitrification, nitrifier denitrification, nitrogen dioxide (NO_2), NO_x cycle.

Abbreviations used: AMO, ammonia mono-oxygenase; AOB, ammonia-oxidizing bacteria.

[1]To whom correspondence should be addressed (email M.J.Kampschreur@tnw.tudelft.nl).

Scheme 1 | **Anoxic (A) and aerobic (B) ammonia oxidation of *Nitrosomonas***

Under anoxic conditions, nitrite is the only electron acceptor available, leading to a high N loss via the denitrification pathway. Under oxic conditions, most electrons are transferred to oxygen and a minor share of the electrons is discharged via denitrification. Adapted from [12] with permission. © 2004 Horizon Scientific Press.



greater significance for the metabolism of ammonia oxidizers. *N. eutropha* in aerobic laboratory-scale cultures was inhibited when gaseous NO was removed from the cultures, by means of intensive aeration, chemical binding with 2,3-dimercapto-1-propanesulphonic acid or removal by *Pseudomonas* PS 88 [10]. Nitrification in these cultures only started again when NO was added.

NO_x seems to be necessary for the start-up of ammonia oxidation in *N. europaea*. The lag phase during recovery of aerobic ammonia oxidation, after growth on hydrogen and nitrite, was significantly reduced when NO_2 was added [11]. Simultaneously, the arrangement of intracytoplasmic membranes changed from circular to flattened vesicles, the protein pattern revealed an increase in the concentrations of 27 and 30 kDa polypeptides, and the cytochrome *c* content increased significantly. When no NO_2 was added, up to 10 p.p.m. NO was detected in the headspace of the cultures. This indicates that the cells generate NO if external NO_x is not available.

Using a continuous aerobic laboratory-scale fermenter with biomass retention, it was shown that NO and NO_2 have a stimulating effect on pure cultures of *N. eutropha*. Compared with cultures grown without these externally added NO_x, there was an increase in specific activity of ammonia oxidation, increased growth rate and increased denitrification capacity. A major part of the ammonia was denitrified to N_2, in the presence of low NO_2 concentrations. The denitrification in the same system, but supplied with NO gas instead of NO_2, was significantly lower [7]. It was hypothesized that, in the presence of NO, AOB elevate their denitrification capacity to compensate for reduced respiration activity, caused by inhibition of cytochrome oxidases by NO [12].

Significance of NO_x in anoxic ammonia oxidation

Under anoxic conditions, between 40 and 60% of the produced nitrite is denitrified to N_2. The specific ammonia oxidation activity is approx. 10-fold lower compared with aerobic conditions due to the fact that only low amounts of NO_2 can be supplemented to the *Nitrosomonas* culture, as high levels are inhibitory [3].

Metabolic activities of *Nitrosomonas europaea* wild-type, and NirK- and NorB-deficient mutants

Three different *N. europaea* strains – wild-type, nitrite reductase (NirK)-deficient and nitric oxide reductase (NorB)-deficient strains – were characterized in chemostat cell cultures, and the effect of NO on metabolic activities was evaluated [13]. All strains revealed similar aerobic ammonia oxidation activities, but the growth rates and yields of the knockout mutants were significantly reduced. N_2 was the main gaseous product of the wild-type, produced via its denitrification activity. The mutants were unable to reduce nitrite to N_2, but excreted more hydroxylamine, leading to the formation of almost equal amounts of NO, N_2O and N_2 by chemical autoxidation and chemodenitrification of hydroxylamine. Under anoxic conditions *N. europaea* wild-type gains energy for growth via NO_2-dependent ammonia oxidation or hydrogen-dependent denitrification using nitrite as electron acceptor. The mutant strains were restricted to NO and/or N_2O as electron acceptor and consequently their growth rates and yields were much lower compared with the wild-type. These findings showed that the denitrification pathway is important for growth of AOB.

Discrimination between aerobic NO_2-dependent and O_2-dependent ammonia oxidation

Batch incubation experiments with and without the addition of acetylene showed that NO_2-dependent and oxygen-dependent ammonia oxidation can be distinguished [14]. Ammonia oxidation by *N. eutropha* with NO_2 as oxidant is not inhibited by acetylene, while oxygen-dependent

ammonia oxidation is inhibited. The labelling reaction of a 27 kDa polypeptide with [^{14}C]acetylene did not occur during anoxic NO_2-dependent ammonia oxidation. When oxygen was added, the labelling of this polypeptide started immediately.

Nitrogen removal by co-culture of *Candidatus* 'Brocadia anammoxidans' and *N. eutropha*

The influence of NO_x was tested on the combination of aerobic and anaerobic AOB [15]. *B. anammoxidans* converts ammonia and nitrite into N_2 and nitrate. In the presence of NO_2, the specific ammonia oxidation activity of *B. anammoxidans* increased, and *Nitrosomonas*-like micro-organisms recovered an NO_2-dependent anoxic ammonia oxidation activity. The anammox bacterium was not inhibited by NO concentrations up to 600 p.p.m. and NO_2 concentrations up to 100 p.p.m. Addition of NO_2 to a mixed population of *B. anammoxidans* and *Nitrosomonas* induced simultaneous specific anaerobic ammonia oxidation activities of up to 5.5 mmol of $NH_4^+ \cdot (\text{g of protein})^{-1} \cdot h^{-1}$ by *B. anammoxidans* and up to 1.5 mmol of $NH_4^+ \cdot (\text{g of protein})^{-1} \cdot h^{-1}$ by *Nitrosomonas*. The stoichiometry of the converted N compounds (NO_2^-/NH_3 ratio) and the microbial community structure were strongly influenced by NO_2. The combined activity of *B. anammoxidans* and *Nitrosomonas*-like ammonia oxidizers might be of relevance in natural environments and man-made ecosystems.

Effect of NO on biofilm formation

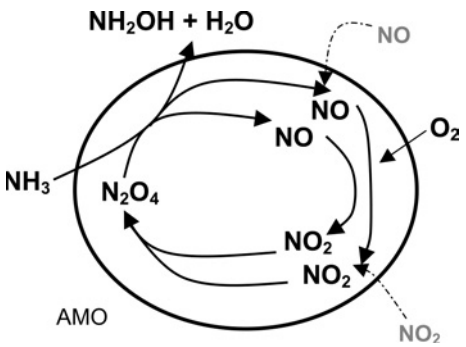
At a NO concentration of more than 30 p.p.m., biofilm formation by *N. europaea* was induced in a continuous aerobic laboratory-scale fermenter [16]. NO concentrations below 5 p.p.m. led to a reversal of the biofilm formation, and the numbers of motile and planktonic (motile-planktonic) cells increased. In a proteomics approach, the six proteins down-regulated by NO in *N. europaea* were identified as flagellar and flagellar assembly proteins.

Hypothetical model of the NO_x cycle during ammonium oxidation

In order to explain the influence of NO_x on ammonia oxidation, a hypothetical model (Scheme 2) was developed [14]. The model summarizes the effects of NO_x on ammonia oxidation, as presented in this paper. N_2O_4 is the oxidizing agent for ammonia oxidation, producing hydroxylamine and NO. Under oxic conditions, NO is reoxidized to NO_2, again providing the AMO with the oxidizing agent (NO_x cycle). Since detectable NO_x concentrations were small, NO_x seems to cycle in the cell (possibly enzyme-bound) and, therefore, the total amount of NO_x per cell is expected to be low. The addition of acetylene leads to an inhibition of aerobic ammonia oxidation, if NO_2 is not present. The cells restart ammonia oxidation when NO_2 is added, and NO is produced in stoichiometric amounts (ratio of NO_2 consumption to NO production is approx. 1:1).

Scheme 2 | NO_x cycle

Hypothetical model of ammonia oxidation by *N. eutropha*. Reproduced from [14] with permission. © 2001 Society for General Microbiology.



Conclusion

NO_x plays an important role in the metabolism of AOB. NO_x enhances recovery of aerobic ammonia oxidation, growth of AOB and denitrification activity. A hypothetical model was developed to explain the influence of NO_x on ammonia oxidation. The exact mechanism behind the stimulating effect of NO_x still needs to be elucidated. The ecophysiological role of NO_x in nitrogen conversion also needs further clarification.

References

1 Abeliovich, A. (1992) Biodegradation **3**, 255–264
2 Weber, S., Stubner, S. and Conrad, R. (2001) Appl. Environ. Microbiol. **67**, 1318–1327
3 Schmidt, I. and Bock, E. (1997) Arch. Microbiol. **167**, 106–111
4 Jiang, Q.Q. and Bakken, L.R. (1999) Appl. Environ. Microbiol. **65**, 2679–2684
5 Shaw, L.J., Nicol, G.W., Smith, Z., Fear, J., Prosser, J.I. and Baggs, E.M. (2005) Environ. Microbiol., doi:10.1111/j.1462-2920.2005.00882
6 Schmidt, I. and Bock, E. (1998) Antonie van Leeuwenhoek **73**, 271–278
7 Zart, D. and Bock, E. (1998) Arch. Microbiol. **169**, 282–286
8 Bock, E., Schmidt, I., Stuven, R. and Zart, D. (1995) Arch. Microbiol. **163**, 16–20
9 Beaumont, H.J.E., Lens, S.I., Reijnders, W.N.M., Westerhoff, H.V. and van Spanning, R.J.M. (2004) Mol. Microbiol. **54**, 148–158
10 Zart, D., Schmidt, I. and Bock, E. (2000) Antonie van Leeuwenhoek **77**, 49–55
11 Schmidt, I., Zart, D. and Bock, E. (2001) Antonie van Leeuwenhoek **79**, 39–47
12 Schmidt, I. and Jetten, M.S.M. (2004) in Strict and Facultative Anaerobes: Medical and Environmental Aspects (Nakano, M.M. and Zuber, P., eds.), pp. 283–301, Horizon Scientific Press, Springer-Verlag, Berlin
13 Schmidt, I., van Spanning, R.J.M. and Jetten, M.S.M. (2004) Microbiology **150**, 4107–4114
14 Schmidt, I., Bock, E. and Jetten, M.S.M. (2001) Microbiology **147**, 2247–2253
15 Schmidt, I., Hermelink, C., Van de Pas-Schoonen, K., Strous, M., op den Camp, H.J., Kuenen, J.G. and Jetten, M.S.M. (2002) Appl. Environ. Microbiol. **68**, 5351–5357
16 Schmidt, I., Steenbakkers, P.J.M., op den Camp, H.J.M., Schmidt, K. and Jetten, M.S.M. (2004) J. Bacteriol. **186**, 2781–2788

Received 22 September 2005