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Metabolism of Inorganic N Compounds by Ammonia-Oxidizing Bacteria

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ABSTRACT: Ammonia oxidizing bacteria extract energy for growth from the oxidation of ammonia to nitrite. Ammonia monooxygenase, which initiates ammonia oxidation, remains enigmatic given the lack of purified preparations. Genetic and biochemical studies support a model for the enzyme consisting of three subunits and metal centers of copper and iron. Knowledge of hydroxylamine oxidoreductase, which oxidizes hydroxylamine formed by ammonia monooxygenase to nitrite, is informed by a crystal structure and detailed spectroscopic and catalytic studies. Other inorganic nitrogen compounds, including NO, N_2O, NO_2, and N_2 can be consumed and/or produced by ammonia-oxidizing bacteria. NO and N_2O can be produced as byproducts of hydroxylamine oxidation or through nitrite reduction. NO_2 can serve as an alternative oxidant in place of O_2 in some ammonia-oxidizing strains. Our knowledge of the diversity of inorganic N metabolism by ammonia-oxidizing bacteria continues to grow. Nonetheless, many questions remain regarding the enzymes and genes involved in these processes and the role of these pathways in ammonia oxidizers.

KEYWORDS: Ammonia monooxygenase, hydroxylamine oxidoreductase, *Nitrosomonas europaea*, nitrite reductase, N cycle, nitrification

INTRODUCTION

Autotrophic ammonia-oxidizing bacteria (AOB) are among a select group of microbes that are able to use ammonia as a sole source of energy and reductant for growth. The product of ammonia oxidation, nitrite, is oxidized to nitrate by the nitrite-oxidizing bacteria as their major source of energy and reductant for growth. Together, these two groups of bacteria carry out the process of nitrification, whereby ammonia is transformed to nitrate. Nitrification, nitrogen fixation, and denitrification comprise the three major processes in the nitrogen (N) cycle. The biogeochemical N cycle involves biological transformations of inorganic N compounds in all ecosystems. The major inorganic N species available for use by organisms are nitrate, ammonia, and dinitrogen. Ammonia, which is released in the decay of organic matter and is a major fertilizer input to croplands, can serve as a nitrogen source for most plants and microorganisms. In some microorganisms, nitrate can serve as an alternative electron acceptor, leading to its reduction to dinitrogen in the process of denitrification. Dinitrogen is the largest N reservoir available to organisms and is reduced to ammonia for biomass production by a limited group of bacteria. Bacteria predominantly, or exclusively, carry out all three processes in the N cycle. The study of these bacteria and the mechanisms by which they transform N compounds has revealed

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a plethora of specialized enzymes, particularly metalloenzymes, which employ a variety of metals (*e.g.*, Fe, Mo, Cu) and utilize novel cofactors and unique chemistries.

Among the elemental biogeochemical cycles, the N cycle has been most influenced by the activities of humans. Anthropogenic input of ammonia into the N cycle primarily through industrially produced fertilizers and cultivation of nitrogen-fixing crops now exceeds the natural contribution (Vitousek *et al.*, 1997). While critical to the intensive agricultural production needed to feed the global human population, these large N inputs can have unintended consequences to the environment. For example, nitrification can lead to detrimental effects in croplands fertilized with ammonia-based fertilizers (*e.g.*, anhydrous ammonia, ammonium salts) or urea (which is degraded to ammonia). The product of nitrification, nitrate, can leach into groundwater and surface water. In groundwater, nitrate can reach concentrations that are unsafe for human consumption. In surface water, excess nitrate upsets the nutrient balance of lakes and streams, leading to eutrophication. From an economic perspective, fertilizer input can be lost to the atmosphere as nitrate is denitrified to dinitrogen (Zumft, 1997). An additional consequence of fertilizer input is the stimulation of nitrifying bacteria to release substantial quantities of N oxides, N_2O and NO, into the atmosphere. N_2O is a potent greenhouse gas (about 200 times more radiatively effective than CO_2), and both NO and N_2O are involved in catalytic ozone destruction in the stratosphere (Stein and Yung, 2003).

In addition to the consequences of nitrification in croplands, nitrification is involved in a number of other important economic and environmental processes. Nitrification initiates the removal of nitrogenous compounds in wastewater and denitrification completes the removal. Some nitrifying bacteria also have potential for remediating environmental pollutants, especially chlorinated aliphatic hydrocarbons, through the process of cometabolism (Arp *et al.*, 2001).

Our goal is to provide a comprehensive review of the metabolism of inorganic N compounds by autotrophic ammonia-oxidizing bacteria. Physiological aspects of N metabolism are considered and the critical enzymes involved plus the genes that encode them are described in detail. We attempted to focus on more recent developments in each of these areas and direct the reader to reviews on specific subjects where appropriate.

AMMONIA-OXIDIZING BACTERIA

Most AOB fall within one taxonomic group in the ß-subclass of the *Proteobacteria*. Seven clusters based on 16S rRNA gene sequences are currently recognized in this group (Kowalchuk and Stephen, 2001). *Nitrosomonas europaea*, a member of cluster 7, is the best studied of the AOB. *N. europaea* grows relatively rapidly and consistently in pure culture. However, it is also one of the most limited AOB physiologically and is not representative of the more dominant groups found in soils.

Ammonia-oxidizing bacteria are fascinating from a physiological standpoint. All are predominantly chemolithoautotrophs (Bock *et al.*, 1991), and for most this appears to be their only growth mode. That is, AOB are incapable of growing heterotrophically. The dependence of AOB on ammonia oxidation is curious given the low energy yield from the oxidation of ammonia ($\Delta G^{\circ\prime} = -271$ kJ mol^{-1}) (Wood, 1986; Hooper, 1989). As autotrophs, nitrifiers typically obtain nearly all of their organic carbon from the assimilation of CO_2. However, chemolithoorganotrophic growth of *N. europaea* with fructose or pyruvate as the C source was recently demonstrated (Hommes *et al.*, 2003). Most nitrifying bacteria produce extensive intracytoplasmic membranes reminiscent of the thylakoids of chloroplasts or the photosynthetic membranes of purple nonsulfur bacteria (Bock *et al.*, 1986). From a metabolic perspective, nitrifiers are among the simplest and most restricted of the autotrophic bacteria. They extract energy from a single inorganic source (NH_3), assimilate inorganic substrates (*e.g.*, CO_2 and NH_3), and use these to synthesize all the necessary biochemicals to support growth.

The complete genome sequence of *N. europaea* has provided new insights into the metabolism of AOB (Chain *et al.*, 2003). The 2.8 Mbp genome includes about 2460 protein-encoding genes. As expected, virtually all of the genes necessary to code for enzymes producing cellular constituents from inorganic nutrients were identified. In contrast, a dearth of genes necessary for the catabolism of organic compounds was notable. The extent of complex repetitive DNA (about 5% of the genome) was surprising. Repeated elements included insertion sequences and Fe acquisition genes. Over 20 genes encoded different classes of Fe siderophore receptors and their regulators. However, no genes for the synthesis of specific siderophores were identified. Apparently, *N. europaea* acquires Fe at the expense of other bacteria that produce siderophores in the environment.

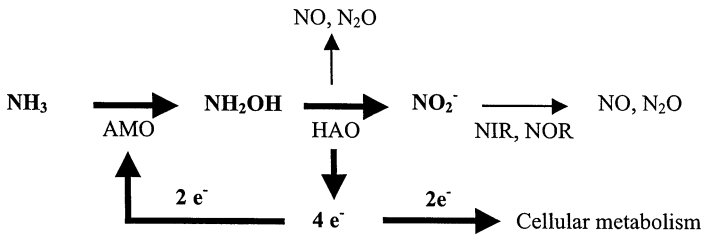

FIGURE 1. Pathway of ammonia oxidation in AOB. Bold arrows and nitrogen compounds indicate major fluxes. AMO, ammonia monooxygenase; HAO, hydroxylamine oxidoreductase; NIR, nitrite reductase; NOR, nitric oxide reductase.

PATHWAY OF AMMONIA OXIDATION

In AOB, the catabolism of ammonia takes place in two steps (Figure 1). Ammonia is first oxidized to hydroxylamine by ammonia monooxygenase (AMO). In monooxygenase-catalyzed reactions, one atom of O from O_2 is reduced with two electrons from the substrate (ammonia in the case of AMO), usually with the insertion of the O atom into the substrate (as in the formation of NH_2OH). There is also a requirement for additional input of reductant to reduce the second atom of O to form H_2O. In AOB, the reductant must come from further oxidation of the product, hydroxylamine. Hydroxylamine is oxidized to nitrite by hydroxylamine oxidoreductase (HAO). This oxidation releases four electrons, two of which are returned to AMO to sustain ammonia oxidation. The remaining two electrons are available for the cell's reductant needs including assimilation of inorganic nutrients and generation of the proton gradient.

Although the predominant nitrogen oxide produced in the process of ammonia oxidation is nitrite, some gaseous products are also formed (Stein and Yung, 2003) (Figure 1). Nitrous oxide and nitric oxide, along with a trace of N_2, have been observed as products of AOB metabolism. Nitrous oxide and nitric oxide can be formed by two routes. First, these are byproducts of the incomplete oxidation of hydroxylamine to nitrite by HAO, which is seen in cell extracts and may also occur in intact cells. Second, both N oxides can be formed by reduction of nitrite in the process of nitrifier denitrification.

BIOENERGETICS OF AMMONIA OXIDATION

While some aspects of proton gradient generation by AOB are understood, other aspects remain enigmatic. From a thermodynamic standpoint, the reduction potential of +0.420 V for the NO_2^-/NH_3 couple is considerably more favorable than +0.772 V for the Fe_3^+/Fe_2^+ couple used by iron-oxidizing bacteria, but considerably less favorable than −0.420 V for the $2H^+/H_2$ couple used by hydrogen-oxidizing bacteria (White, 2000). However, based on our current knowledge of ammonia-oxidizing metabolism, only electron flow from hydroxylamine to O_2 is expected to contribute to proton gradient formation (published values for the $E^{o'}$ for NO_2^-/NH_2OH redox couple are +0.060 and +0.127 V) (Wood, 1986; Poughon *et al*., 2001) and only half of the electrons released in the oxidation of hydroxylamine are available for energy needs of the cell because the other half must be returned to AMO to sustain ammonia oxidation (Whittaker *et al*., 2000; Poughon *et al*., 2001).

Understanding the bioenergetics of AOB requires knowledge of (1) the pathway of electron flow as ammonia is oxidized to nitrite, (2) the contributions to the proton gradient, and (3) proton dissipation through two major sinks: ATP synthase and NADH oxidoreductase (for NAD^+ reduction via reverse electron flow). To a first approximation, electron flow in AOB involves a "typical" bacterial four-component electron transport chain with the overlay of ammonia oxidation components. However, the components may not all function as in the mitochondrial model (Wood, 1986; Whittaker *et al*., 2000; Poughon *et al*., 2001).

The redox potential for the oxidation of ammonia to hydroxylamine coupled to the reduction of O_2 to H_2O is +0.8 to 0.9 V (Wood, 1986; Poughon *et al*., 2001). In theory, electrons required for this monooxygenase reaction could come from any point in the electron transport chain. Current thinking holds that AMO draws electrons from the ubiquinol pool, perhaps with ubiquinol as the direct electron donor (Figure 2). This idea is supported by the observation that tetra- and tri-methylhydroquinols can support AMO activity in cell extracts (Shears

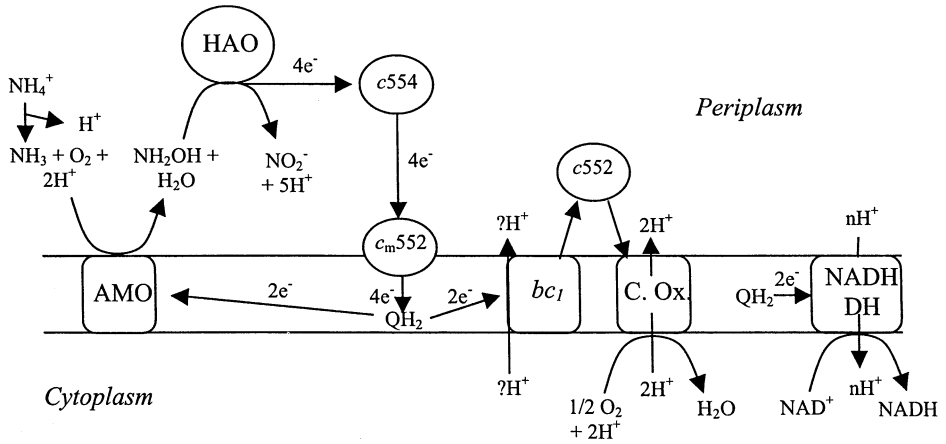

FIGURE 2. Model of electron flow in *N. europaea*. AMO, ammonia monooxygenase; HAO, hydroxylamine oxidoreductase; bc_1, Complex III; C. Ox., cytochrome oxidase; NADH DH, NADH dehydrogenase; *c*, cytochrome; QH_2, quinol.

and Wood, 1986). Hydroxylamine oxidation serves as an important node (branchpoint) in the flow of electrons. All four electrons likely pass through cyt *c*554, which was shown to accept electrons from HAO *in vitro* (Yamanaka and Shinra, 1974). Most of the electron flux is expected to flow through the ubiquinone pool. From cyt *c*554, the simplest path to ubiquinone would be through cyt c_m552 (Whittaker *et al*., 2000). The ubiquinone pool serves as another important node. Half of the electron flux, or 2 moles of electrons for every mole of hydroxylamine oxidized, must return to AMO to sustain ammonia oxidation. Most of the rest of the electrons flow to O_2. Whittaker *et al*. (2000) showed that inhibitors of electron transfer through cytochrome bc_1 (Complex III) also inhibited NH_3 and NH_2OH oxidation and concluded that electrons from the oxidation of hydroxylamine pass through this complex. From the cyt bc_1 complex, electrons pass through cytochrome oxidase to O_2. The soluble periplasmic cytochrome *c*552 may facilitate this final step (DiSpirito *et al*., 1986). A small flux of electrons from the ubiquinone pool (6–12%) must be used in the reduction of NAD^+ (Wood, 1986; Poughon *et al*., 2001). Because the direction of electron flow is "uphill" thermodynamically with regard to the midpoint potentials of the ubiquinone/ubiquinol couple (+0.050 to 0.100 V) and the NAD^+/NADH couple (−0.320 V), this flux is referred to as "reverse electron flow." By using NADH oxidoreductase (Complex I) in reverse, the cell can take advantage of the proton motive force (173 mV) to help close the gap in midpoint potentials (Wood, 1986; Poughon *et al*., 2001). Maintenance of a high ratio of ubiquinol to ubiquinone (requiring tight respiratory control) further closes the gap. As pointed out by Poughon *et al*. (2001), reduction of NAD^+ is expected to occur near equilibrium for NAD^+ and NADH.

Energy released from hydroxylamine oxidation is conserved in a proton motive force, consisting of a proton gradient across the cytoplasmic membrane. The force has been estimated at 173 mV (Kumar and Nicholas, 1982). Protons are translocated across the membrane at cytochrome oxidase ($1H^+/e^-$, $2H^+/NH_3$). Using a metabolic flux analysis, Poughon *et al*. (2001) argued that proton translocation was not occurring at the level of the Q cycle/bc_1 complex. If correct, then the $2H^+/e^-$ ($4H^+/NH_3$) expected in this reaction are not translocated, even though electrons are passing through the complex. In contrast, Whittaker *et al*. (2000) proposed that protons are translocated as a result of electron flow through the bc_1 complex, which is how most bacterial bc_1 complexes operate. Further experiments are required to resolve this question. Scalar protons (those generated or consumed during reactions associated with cellular metabolism) can either contribute to or diminish the proton gradient. The major source of scalar protons is from the transformation of hydroxylamine and water to nitrite with the release of five protons into the periplasmic space ($5H^+/NH_3$). In the case of ammonia oxidation by AMO, two protons are consumed in the formation of water. AMO is localized in the cytoplasmic membrane, but it is not known if ammonia is oxidized on the periplasmic or cytoplasmic side of the membrane. Oxidation in the periplasm would have the advantage that toxic hydroxylamine remains in the periplasm (as shown in Figure 2) and in the same compartment as HAO. However,

the consumption of two protons from the periplasm would diminish the proton gradient. Oxidation of ammonia and consumption of protons on the cytoplasmic face would have the advantage of contributing to the proton gradient, but would have the considerable disadvantage of the need for ammonia transport into the cytoplasm and hydroxylamine transport into the periplasm. In the production of H_2O by cytochrome oxidase, an additional two H^+ are consumed in the cytoplasm. Finally, ammonia will come primarily from ammonium, the dominant partner in the equilibrium at environmentally relevant pH values. Assuming that ammonium enters the periplasm, another scalar proton will be contributed to the gradient with each ammonia molecule oxidized. Proton translocation was demonstrated experimentally for hydroxylamine oxidation (1 mM) with a H^+/O ratio of 4.0 and for ammonium (1 mM and 10 mM) with ratios of 3.1 and 1.3, respectively (Drozd, 1976; Hollocher *et al*., 1982).

AMMONIA OXIDATION

Ammonia Monooxygenase Structure

Ammonia monooxygenase (AMO) has not yet been purified extensively with activity, in spite of considerable effort (Suzuki and Kwok, 1970; Suzuki *et al*., 1981; Ensign *et al*., 1993; Juliette *et al*., 1995). Therefore, much of our knowledge of AMO has been deduced from experiments with intact cells or cell extracts. AMO is catalytically, structurally, and genetically similar to particulate methane monooxygenase (pMMO) of methanotrophs (Holmes *et al*., 1995; Semrau *et al*., 1995; Murrell and Holmes, 1996; Nguyen *et al*., 1998). More progress has been made in purifying and characterizing the pMMO system, so we take many clues from pMMO in describing AMO (Zahn and DiSpirito, 1996; Nguyen *et al*., 1998; Basu *et al*., 2003; Lieberman *et al*., 2003). AMO has a broad substrate range and inhibitor profile, and these have also provided insights into both binding sites and catalytic mechanism (Bedard and Knowles, 1989; Hooper *et al*., 1997). Most notably, use of radiolabeled acetylene, a mechanism-based inactivator of AMO, led to the initial identification of the catalytically active AMO polypeptide (Hyman and Wood, 1985).

AMO and pMMO were long considered to be in a monooxygenase class by themselves based on their inhibitor profiles and protein compositions. However, Hamamura and Arp (2000) identified a butane monooxygenase (pBMO) in a butane-grown bacterium that shares similar inhibitor and inactivator profiles with AMO and pMMO, is inactivated by exposure to visible light as is AMO (Hooper and Terry, 1974), is membrane bound, and consists minimally of a 27 kDa polypeptide that is labeled when butane uptake is inactivated by treatment with $^{14}C_2H_2$. Identified in a *Nocardioides* species, this is the first example of a monooxygenase in a gram-positive bacterium that shares similar properties with AMO and pMMO (Hamamura and Arp, 2000). While all three enzymes—AMO, pMMO, and pBMO—oxidize butane and ammonia, pBMO apparently cannot oxidize methane, as can AMO (Hyman and Wood, 1983) and pMMO. An AMO purified from the heterotrophic AOB *Paracoccus denitrificans* (Moir *et al*., 1996) shows some similarities to AMO from autotrophs. For example, the enzyme contains labile Cu and consists of two subunits (36 and 46 kDa) with masses similar to those of AMO. However, acetylene did not inactivate the *P. denitrificans* enzyme and oxidation of alternative substrates was not reported.

Three lines of evidence have led to the conclusion that AMO contains Cu: (1) inhibition by Cu-selective chelators (Hooper and Terry, 1973; Bedard and Knowles, 1989); (2) restoration of activity in cell extracts by treatment with Cu (Ensign *et al*., 1993); and (3) by analogy with pMMO where active, purified preparations contain Cu (Zahn and DiSpirito, 1996; Nguyen *et al*., 1998; Basu *et al*., 2003; Lieberman *et al*., 2003). There is also evidence for Fe in AMO (Zahn *et al*., 1996). Again, it should be noted that purified preparations of AMO with strong activity are not yet available. Without such preparations, the precise metal content remains a topic of speculation.

AMO likely consists of three polypeptides, AmoA, AmoB, and AmoC, all of which are membrane bound. The evidence for these three polypeptides is biochemical, genetic, and through analogy with pMMO. AmoA covalently binds $^{14}C_2H_2$, which led to its identification in extracts as a 27 kDa polypeptide (Hyman and Wood, 1985). This size is somewhat smaller than that (31,990 Da) predicted by the gene for AmoA (*amoA*) (McTavish *et al*., 1993). However, it is not unusual for membrane-bound proteins to migrate on SDS-PAGE at rates inconsistent with their actual size. AmoB is encoded by *amoB* (McTavish *et al*., 1993; Bergmann and Hooper, 1994). The *amoB* gene predicts a polypeptide with a mass of 44,266 Da following cleavage of a periplasmic signal peptide, consistent with the

experimentally determined size of 43 kDa (Bergmann and Hooper, 1994). AmoB copurifed with AmoA following treatment of cells with an alkyne-based inactivator and is likely part of a complex with AmoA (McTavish *et al*., 1993). Biochemical evidence defining a role for AmoC is scant. Genetic evidence of a contiguous operon structure supports a complex with all three polypeptides (Sayavedra-Soto *et al*., 1996, 1998), as does analogy with pMMO, which can be purified as a $\alpha_2\beta_2\gamma_2$ complex of PmoB, PmoA, and PmoC (Zahn and DiSpirito, 1996; Nguyen *et al*., 1998; Basu *et al*., 2003; Lieberman *et al*., 2003). However, purified pMMO complexes with only PmoA and PmoB have also been obtained, albeit without activity (Zahn and DiSpirito, 1996).

Genes Encoding AMO

The genes encoding AMO have been sequenced from a number of ammonia oxidizing bacteria (Bergmann and Hooper, 1994; Klotz and Norton, 1995; Arp *et al*., 2002; Norton *et al*., 2002). As expected, the strongest matches to the *N. europaea amo* genes in the nucleotide sequence databases, besides other ammonia oxidizers, are the corresponding genes from methanotrophs (Holmes *et al*., 1995). As with genes encoding pMMO, the genes encoding AMO are in an ordered cluster of *amoC-amoA-amoB*. A unique feature of this cluster is that it is present in two to three copies in most AOB (McTavish *et al*., 1993; Klotz and Norton, 1995; Sayavedra-Soto *et al*., 1998; Norton *et al*., 2002), although only a single copy is present in *Nitrosococcus oceani* (a γ-proteobacterium) (Alzerreca *et al*., 1999). The copies within each organism are nearly identical, while the sequences are divergent among organisms. This result suggests that the cells have a mechanism for rectifying or reconciling differences between copies within an organism (Klotz and Norton, 1998). Disruptions of individual copies of *amoA* or *amoB* in *N. europaea* were not lethal, suggesting that all copies were functional and sufficient for growth (Hommes *et al*., 1998). During growth or upon transfer of cells to fresh medium, and in spite of identical promoters for each copy small differences in the expression of the copies were revealed in mutants with one copy disrupted (Hommes *et al*., 1998, 2001; Stein *et al*., 2000).

An open reading frame (ORF 4) is present downstream from both copies of *amoB* in *N. europaea* (Chain *et al*., 2003) and several other AOB (Alzerreca *et al*., 1999; Norton *et al*., 2002). Perhaps this ORF encodes a protein that is involved in ammonia oxidation. The sequence predicts a protein product with a mass of 23,535 Da that includes two transmembrane helices, but the sequence does not provide much insight as to its possible role. A standalone copy of *amoC* is also present in the genome (Sayavedra-Soto *et al*., 1998). This copy is somewhat divergent from the copies in the *amoCAB* clusters. Again, its function is unknown.

The *amoCAB* cluster does constitute an operon. In *N. europaea*, transcripts encoding AmoCAB were observed (Hommes *et al*., 2001). However, transcripts encoding AmoC alone and only AmoAB were also observed and may have been derived from the full length transcript or transcribed independently. Independent expression of *amoA* in *Nitrosospira* sp. NpAV was observed by heterologous expression of *amoA* in *Escherichia coli* (Norton *et al*., 1996). These results indicate that at least two separate promoters, one controlling *amoC* and one controlling *amoAB* expression, are also present within the *amoCAB* operon.

Catalytic Function of AMO

The roles of each of the three AMO polypeptides in ammonia oxidation are not well defined. The labeling of AmoA by the mechanism-based inactivator, $^{14}C_2H_2$, has led to the conclusion that this subunit contains the site of substrate oxidation (Hyman and Wood, 1985). However, the roles of AmoB and AmoC are unknown. One of the subunits might be involved in transferring reductant to the active site, but there is no experimental evidence to allow assignment of this role to a particular subunit. A detailed analysis of the deduced sequences for each of the subunits also does not provide much insight into their roles. Six transmembrane alpha helixes are predicted in AmoA, two in AmoB, and another six in AmoC. No motifs were recognized in any of the sequences with the sequence motif search program MOTIF (http://motif.genome.ad.jp/). For example, neither quinone- nor copper-binding sites were observed. Clearly many questions about the structure and function of this enigmatic enzyme remain unanswered, such as the structure of the acetylene adduct and its point of attachment to AMO. The binding site for the electron donor to AMO remains unknown, as does the binding site for the presumed associated metals.

The evidence that ammonia is catabolized by a monooxygenase reaction came from labeling (Hollocher *et al*., 1981) and inhibitor (Hofman and

Lees, 1953) studies. One molecule of O_2 is consumed directly with the oxidation of one molecule of ammonia. Overall, one atom from O_2 is inserted into ammonia to form the product, hydroxylamine, while the second atom is reduced ($O^=$) and picks up two protons to form water. This reduction requires an input of two electrons per molecule of ammonia oxidized or O_2 reduced. While the source of reductant has not been identified positively, accepting reductant from the quinone pool, either directly or via an electron mediator, seems most likely and is consistent with experimental results (Shears and Wood, 1986). A low potential reductant, such as NADH, is not required for the reaction. While NADH can support activity in cell extracts (Suzuki *et al*., 1981), it is not likely to serve as the reductant *in vivo* because the NO_2^-/NH_2OH couple is about 300 mV more positive than the NAD^+/NADH couple. In these obligate lithotrophs, the NO_2^-/NH_2OH couple is the ultimate source of reductant for all cellular reactions.

Ammonia, not ammonium, is the substrate for AMO (Suzuki *et al*., 1974). The K_s for ammonium (the K_m measured in intact cells) is about 1.3 mM at pH 7.7 (Keener and Arp, 1993), which corresponds to a K_s of 46 μM ammonia considering the pK of 9.25 (25°C) for the dissociation of ammonium to ammonia and H^+. This K_s is in good agreement with the value of 20 μM determined by Suzuki *et al*. (1974) for cell free extracts. In *N. europaea*, AMO has a remarkably broad substrate range (Hooper *et al*., 1997). While ammonia is the only substrate for AMO that can support growth, a variety of compounds can find their way into the active site and undergo O insertion reactions, or in a few cases dehydrogenations or reductive dehalogenations (Table 1). A variety of inhibitors and inactivators of AMO have been identified (Table 2) (Hooper and Terry, 1973; Hyman *et al*., 1988; Bedard and Knowles, 1989; Keener and Arp, 1994; Keener, 1995; Keener *et al*., 1998). Most significant among the inactivators is acetylene.

TABLE 1
Examples of Substrates Transformed by AMO and the Resulting Products

Substrates	Products	References
O-Insertion Reactions		
Ammonia	*Hydroxylamine*	Hofman and Lees, 1953
Alkanes	Alcohols	Hyman and Wood, 1983;
Methane	*Methanol*	Hyman *et al*., 1988
Butane	1-, 2-*butanol*	
Alkenes	Epoxides	Hyman and Wood, 1984;
Ethene	*Ethylene oxide*	Hyman *et al*., 1988
Propene	*Propylene oxide*	
Aromatic hydrocarbons	Alcohols	Hyman *et al*., 1985; Vannelli
Benzene	*Benzyl alcohol*	and Hooper, 1995; Chang
Naphthalene	*Naphthol*	*et al*., 2002
Thioethers	Sulfoxides	Hyman *et al*., 1985; Juliette
Dimethylsulfide	*Dimethylsulfoxide*	*et al*., 1993a; Vannelli and Hooper, 1995; Chang *et al*., 2002
O-Ethers	Hydrolysis products	Hyman *et al*., 1994
Dimethyl ether	*Methanol and formaldehyde*	
Halogenated Compounds	Various compounds	Hyman and Wood, 1984a;
Bromoethane	*Acetaldehyde and* Br^-	Rasche *et al*., 1990;
Chlorobenzene	*4-chlorophenol*	Vannelli *et al*., 1990;
Trichloroethylene	*TCE-epoxide (?)*	Keener and Arp, 1994
Dehydrogenation Reaction		
Ethylbenzene	*Styrene*	Keener and Arp, 1994
Reductive Dehalogenation		
2-chloro-6-trichloromethyl-pyridine (nitrapyrin)	*2-chloro-6-dichloromethyl-pyridine*	Vannelli and Hooper, 1993

TABLE 2
Inhibitors and Inactivators of AMO

Type	Examples	Comments	References
Inhibitors			
Competitive vs. NH_3	Methane, ethylene, carbon monoxide	Alternative substrates	Suzuki *et al*., 1976; Keener and Arp, 1993
Noncompetitive vs. NH_3	Ethane, chloroethane	Alternative substrates	Keener and Arp, 1993
Metal chelators	Thiourea	Cu-selective	Hooper and Terry, 1973
Inactivators			
Mechanism-based	Alkynes, allylsulfide, p-anisidine	Require enzyme turnover with O_2	Hynes and Knowles, 1978; Hyman et al., 1988; Juliette *et al*., 1993b; Keener *et al*., 1998
Unknown	Nitrite	NH_3 and alkanes protect, O_2 not required	Stein and Arp, 1998

As a mechanism-based inactivator, inactivation by acetylene requires turnover conditions (Hyman and Wood, 1985). As AMO oxidizes acetylene, a highly reactive product then reacts with AMO, resulting in a covalent bond between AMO and the acetylene product. Alkynes and other compounds also appear to be mechanism-based inactivators (Table 2) (Hyman *et al*., 1988; Keener *et al*., 1998). However, not all the criteria for mechanism-based inactivators are met by each of these compounds (Silverman, 1988). For example, the rate of inactivation should reach a maximum when the concentration of inactivator (I) is high enough to drive all available enzyme (E) into the E·I complex. This criterion was met for *p*-anisidine, but not for any alkynes tested, including acetylene (Keener *et al*., 1998). The natural substrate, in this case ammonia, should protect monooxygenases from inactivation. While this is the case for acetylene, ammonia actually stimulated the rate of inactivation with other alkynes and most other inactivators (Juliette *et al*., 1993b; Keener *et al*., 1998). Nitrite can inactivate AMO in *N. europaea* in the presence or absence of O_2, although the mechanism is unclear (Stein and Arp, 1998). Ammonia and short chain alkanes protect AMO from inactivation by nitrite, which explains why cells grow in batch cultures where concentrations of nitrite reach 10–20 mM but only when ammonia is present in excess (Stein and Arp, 1998). Clearly, the precise mechanisms by which several of these compounds inactivate AMO remain to be determined.

Alternative substrates of AMO are also inhibitors. For example, methane inhibits the oxidation of ammonia (Suzuki *et al*., 1976). While one might expect all of the alternative substrates to be competitive inhibitors of ammonia oxidation (competing for the same binding site), a kinetic analysis revealed that while some alternative substrates were competitive inhibitors, others were noncompetitive, implying that an Enzyme · Inhibitor · Substrate complex was possible (Keener and Arp, 1993). Perhaps the alternative substrate and ammonia were competing for alternative binding sites within the same active site. Some metal chelators are also inhibitors of AMO (Bedard and Knowles, 1989). Most useful among these have been the thiourea-based compounds (*e.g*., allylthiourea), which are copper-selective chelators (Hooper and Terry, 1973).

Like other aspects of our understanding of AMO, a thorough description of the catalytic mechanism will require detailed spectroscopic and kinetic studies with purified enzyme. From the suite of substrates and inhibitors, some general trends can be observed and a reaction mechanism envisioned (Wood, 1986; Hooper *et al*., 1997; Keener *et al*., 1998). All the substrates (including ammonia) are uncharged and nonpolar or of relatively low polarity. This observation argues for a hydrophobic substrate-binding pocket. By analogy with other metal-containing monooxygenases, it seems reasonable to expect O_2 to bind to one of the metals (Cu or Fe) where the O_2 may be activated by a two-electron transfer from the metal(s). A reduced $O^{=}$ species would be eliminated with formation of H_2O. For most substrates, this activated M = O intermediate in the active site would then extract a hydrogen, leading to formation of a metal-bound hydroxyl and a substrate radical. Rebound of the hydroxyl

onto the substrate radical would lead to formation of hydroxylated products. Variations on this general theme are required to account for the formation of epoxides and for the dehydrogenation and reductive dehalogenation reactions. The broad substrate range also argues for binding and activation of O_2 followed by binding of substrate.

O_2-INDEPENDENT, NO_2-LINKED AMMONIA OXIDATION

Ammonia oxidation was considered a strictly aerobic process until the last few years when two seminal discoveries challenged this idea. First, a process known as anammox was discovered in which bacteria can grow anaerobically at the expense of ammonia oxidation with nitrite as the electron acceptor and with the formation of N_2 (Jetten *et al*., 1999; Schmidt *et al*., 2002) (Scheme 1). Hydrazine appears to be an intermediate, and a HAO-like enzyme is involved in its conversion to N_2. The mechanism by which ammonia is combined with nitrite to form hydrazine is unknown. The anammox process is outside the scope of this review, but several reviews of the process are available (Jetten *et al*., 1999; Jetten *et al*., 2001; Schmidt *et al*., 2002). Second, Bock and colleagues have shown that *Nitrosomonas eutropha*, an aerobic ammonia-oxidizing bacterium, can oxidize ammonia anoxically with NO_2 as the electron acceptor (Schmidt and Bock, 1997) (Scheme 1). Under anoxic conditions, ammonia reacts stoichiometrically with 2 molecules of NO_2 (or one molecule of its dimeric form, N_2O_4, which dominates the equilibrium at 25°C) to form NH_2OH and two molecules of NO (Schmidt and Bock, 1997; Schmidt and Bock, 1998; Schmidt *et al*., 2002). Like the analogous reaction with O_2, there is a requirement for reductant (2 e^-) to complete the reduction of the second molecule of NO_2 (or the second half of N_2O_4) to NO. The 2:1 stoichiometry is somewhat surprising given that a 1:1 stoichiometry would be more efficient by avoiding the need to recycle half the reductant released in the subsequent oxidation of hydroxylamine back to AMO to reduce the second molecule of NO_2.

$$NH_3 + 1.5\ O_2 \rightarrow NO_2^- + H_2O + H^+ \quad \text{(a)}$$

$$3\ NH_3 + 3\ N_2O_4 \rightarrow NO_2^- + 6\ NO + N_2 + 4\ H_2O + H^+ \quad \text{(b)}$$

$$NH_3 + NO_2^- \rightarrow N_2 + H_2O + OH^- \quad \text{(c)}$$

SCHEME 1. Aerobic and anaerobic ammonia oxidation. (a) Aerobic ammonia oxidation by ammonia-oxidizing bacteria. (b) Anaerobic ammonia oxidation by the ammonia-oxidizing bacterium, *N. eutropha*. (c) Anaerobic ammonia oxidation by anammox bacteria.

Based on several lines of evidence with *N. eutropha*, a "NO_x" cycle was proposed to function under oxic conditions (Schmidt *et al*., 2002). In this model, NO produced in the reaction of ammonia with NO_2 is reoxidized to NO_2 in a reaction with O_2. It is not clear if the NO_x cycle would be the preferred mechanism of ammonia oxidation (*i.e*., NO_2, rather than O_2, is the predominant oxidant *in vivo*), a minor side reaction, or if both can function simultaneously and at similar rates. Vigorous purging with air (to remove NO) resulted in decreased rates of nitrite production until NO was introduced into the gas stream (Zart *et al*., 2000). Starvation recovery lags were also reduced when cells were treated with NO (Schmidt *et al*., 2001b). A central experiment involved the demonstration that acetylene did not inactivate NO_2-dependent NO_2^- production, though it did inactivate O_2-dependent NO_2^- production (Schmidt *et al*., 2001a). Likewise, AmoA was only labeled by $^{14}C_2H_2$ in the presence of O_2. The requirement of O_2 for labeling and inactivation was expected, given previous work with *N. europaea* (Hyman and Wood, 1985). The combined results were interpreted as a single AMO enzyme responsible for both O_2- and NO_2-dependent ammonia oxidation activities (Schmidt *et al*., 2001a). However, another interpretation is that a second type of ammonia-oxidizing enzyme is present in *N. eutropha* that utilizes NO_2 as the oxidant and is not responsive to acetylene. Indeed, in the critical experiment treating the NO_2/O_2 sample with acetylene, a residual ammonia oxidizing activity of about 10% was present. In contrast, ammonia oxidation was completely inactivated by acetylene when O_2 was the sole oxidant. Furthermore, the rate of ammonia oxidation with NO_2 as the sole oxidant was about 10% of the rate with O_2 as the sole oxidant. Another surprising result was that NO protected O_2-dependent ammonia oxidation activity from inactivation by acetylene. The authors speculated that acetylene and NO might compete for a binding site that is used primarily for O_2-dependent NO oxidation to NO_2 (Schmidt *et al*., 2001a). In this case, O_2 and NO_2 would bind to separate catalytic sites on AMO. An alternative explanation is that O_2, NO, and acetylene bind to the same active site such that NO protects the enzyme from inactivation by acetylene by preventing O_2 binding and catalytic turnover.

In thinking about the role of NO_2 as an oxidant for ammonia oxidation, a number of additional questions arise. How common is this ability among the AOB? Can, or must, NO_2 serve as the oxidant for the many alternative substrates of AMO or is the reaction of NO_2 with ammonia unique? Is there a strong NO/NO_2 cycle in nature to make this a significant metabolism? Methane monooxygenases (Dalton, 1977) and several butane monooxygenases (Hamamura *et al*., 1999) can also oxidize ammonia to hydroxylamine. Are these monooxygenases also capable of using NO_2 as an oxidant, and if so, is this ability limited to, or required for, the oxidation of ammonia? Regardless of the answers to these questions, the complex reactions of *N. eutropha* with nitrogen oxides, both aerobically and anaerobically, considerably expands the known limits of metabolism of inorganic N compounds by ammonia-oxidizing bacteria.

HYDROXYLAMINE OXIDATION

Hydroxylamine Oxidoreductase Structure

HAO catalyzes the oxidation of hydroxylamine to nitrite. HAO is a remarkable enzyme. It is an α_3-trimer of a 60 kDa polypeptide (Igarashi *et al*., 1997). Each polypeptide harbors eight c-type hemes, for a total of 24 hemes/enzyme (Arciero *et al*., 1993a). One of these eight hemes is unique in that it has an additional covalent attachment to the protein through a tyrosine residue (Arciero *et al*., 1993b). Interestingly, this third point of attachment is to a different subunit than the two cysteinyl thiol linkages (Igarashi *et al*., 1997). This unique heme also has altered spectral properties (Soret absorbance at 463 nm in the reduced state, hence referred to as the P460 chromophore) and is the active site of HAO (Hooper *et al*., 1997). Some of the additional hemes presumably function to funnel electrons out of the active site to the electron carriers (Igarashi *et al*., 1997). The midpoint potentials of the hemes vary from +288 to −412 mV; the midpoint potential for P460 is −260 mV (Collins *et al*., 1993).

The crystal structure of HAO revealed the location of each of the hemes in the trimer. Within each subunit, the hemes are arranged into four groups (Igarashi *et al*., 1997). Hemes P460 (−260 mV), 6 (−190 mV), and 7 (−150 mV) form a cluster, hemes 1 and 2 constitute a cluster, hemes 3 (0 mV) and 5 (0 mV) constitute the third cluster, and heme 8 stands alone (Hendrich *et al*., 2001) (Figure 3). The midpoint potentials associated with hemes 1, 2, and 8 are +288 mV, −265 mV, and −412 mV, but these potentials have not yet been matched with their heme. The triheme cluster forms the site of hydroxylamine oxidation. Based on the organization of hemes in the enzyme, Igarashi *et al*. (1997) envisioned two possible routes of electron flow from the triheme cluster. Electron flow through the heme 3–5 cluster and on to the heme 1–2 cluster was proposed as the major route of electron flow. A minor route would be through heme 8. Heme 8 is located such that electron flow to the heme 1–2 cluster of an adjacent subunit seems possible with the consequence that the subunits would not necessarily be functioning independently. The known assignments of midpoint potentials are consistent with the proposed major pathway. It will be interesting to determine which of the hemes carries the low midpoint potential (−412 mV). This low potential would seem to disfavor a pathway incorporating this heme.

HAO as described above is essentially unique to autotrophic AOB in terms of overall structure, catalytic activity, and primary sequence. For example, hydroxylamine oxidation in heterotrophic bacteria is catalyzed by distinct enzymes such as the 20 kDa nonheme iron protein from *P. denitrificans* (Moir *et al*., 1996) or the 68 kDa homo-dimer with no detectable cofactors from *Pseudomonas* PB16 (Jetten *et al*., 1997). However, an enzyme with some striking similarities to HAO was purified from an anammox enrichment culture (Schalk *et al*., 2000). The purified protein is an α_3 trimer composed of 58 kDa subunits, contains 26 ± 4 c-type hemes, exhibits a P468 chromophore, and catalyzes the oxidation of hydroxylamine to nitrite. Nonetheless, sequences of four peptide fragments were not similar to known HAO sequences, significant spectral differences were observed, and significant catalytic differences were identified. In spite of the uniqueness of HAO, structural studies have revealed some conservation of the underlying heme organization. The arrangement of hemes 4, 6, 5, and 3 in *N. europaea* HAO (Igarashi *et al*., 1997) shows a remarkable similarity to the structural arrangement of the four hemes in *c*554 from *N. europaea* (Iverson *et al*., 2001) and to four hemes of the pentaheme cytochrome *c* nitrite reductases from *Sulfurospirillum deleyianum* (Einsle *et al*., 1999), *Wolinella succinogenes* (Einsle *et al*., 2000) and *E. coli* (Bamford *et al*., 2002), which catalyze the reduction of nitrite to ammonium.

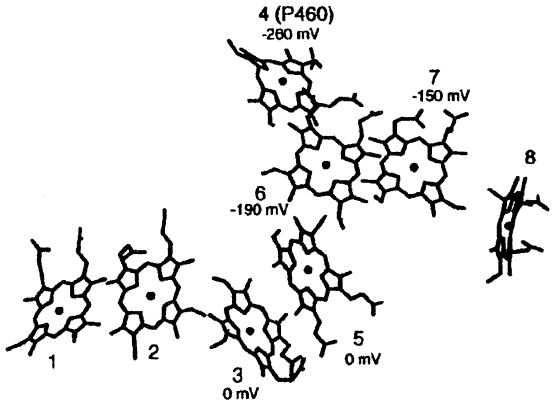

FIGURE 3. Spatial arrangement of hemes from the crystal structure in a monomeric unit of HAO. The heme numbering scheme and assignments of various heme midpoint potentials are shown. Reprinted with permission from Hendrich *et al.* (2002). Copyright 2002 American Chemical Society.

Genes Encoding HAO, *c*554, c_m552

The gene encoding HAO (*hao*) is monocistronic (Sayavedra-Soto *et al*., 1994). Like the genes for AMO, the genes for HAO are present in multiple copies with three copies in *N. europaea* (McTavish *et al*., 1993). The deduced amino acid sequence of HAO reveals a periplasmic leader sequence consistent with the experimentally determined location of the protein (Sayavedra-Soto *et al*., 1994). Unlike AMO, where motif searches yield no insights, all eight heme-binding sites are readily identified in the gene sequence. The genes for *c*554 (*hcy* or *cycA*) are located downstream from all three *hao* copies (Bergmann *et al*., 1994; Hommes *et al*., 1994; Sayavedra-Soto *et al*., 1994). For two of the three *hcy* copies the genes for c_m552 are contiguous, but the third copy of *hcy* is not accompanied by the gene for c_m552 (Arp *et al*., 2002).

Biochemical Evidence for NO Production During the Catalytic Cycle of HAO

Although HAO was purified to homogeneity from *N. europaea* several years ago, the complete catalytic cycle of hydroxylamine oxidation to nitrite remains unclear (Hooper and Terry, 1977). One of the critical questions is whether NH_2OH oxidation involves a NO intermediate, as in Scheme 2.

$$NH_2OH \rightarrow HNO \rightarrow NO \rightarrow NO^+ \rightarrow HNO_2$$

SCHEME 2.

Evidence in favor of NO formation during NH_2OH oxidation includes the production of NO and N_2O in growing cultures of *N. europaea* (Hynes and Knowles, 1984), *in vitro* oxidation of NH_2OH to HNO (Hooper and Terry, 1979), and the anaerobic production of NO and N_2O from NH_2OH by purified HAO from an anammox enrichment culture (Schalk *et al*., 2000). None of these reactions were attributed to the reduction of nitrite, but rather to the direct catalytic mechanism of HAO actively oxidizing NH_2OH. For HAO to produce NO from NH_2OH, a single electron transfer from one heme group to another is required, whereas successive two-electron transfers would bypass a NO intermediate. Evidence for two-electron transfers are indirectly supported by the inability of purified HAO to oxidize exogenously provided NO to nitrite (Hendrich *et al*., 2002).

To address the issue of whether a NO intermediate is formed during NH_2OH oxidation, two recent studies demonstrated interactions between the P460 active-site of purified HAO and NO (Hendrich *et al*., 2002; Cabail and Pacheco, 2003). The first study showed that exogenously added NO binds to heme Fe within HAO between P460 and heme 6, forming a diamagnetic $\{FeNO\}^6$ complex (Hendrich *et al*., 2002). Oxidized HAO was recovered after gas exchange and exposure to light, a required process due to the very tight binding of NO to heme Fe. The authors concluded that NO is not a likely intermediate in NH_2OH oxidation to nitrite because: (1) unlike other heme-containing proteins the addition of NO resulted in the oxidation of HAO (and the likely concomitant reduction of NO to N_2O), perhaps due to the low redox potential of P460 (midpoint potential $= -260$ mV); (2) in the presence of high

concentrations of NH_2OH the natural yield of NO-ferrous heme complex was low; and (3) the coordination of P460 to a tyrosine residue and its association with seven other electron-accepting hemes (Igarashi *et al*., 1997) should favor the removal of electrons two by two rather than single electron transfers. The authors also indicated that the tight association of NO with P460 would be problematic if abundant NO were produced normally during NH_2OH oxidation.

In the second study, the authors reached a contradictory conclusion, namely that HAO is reduced by NO through a one electron transfer reaction concomitant with the oxidation of NO to nitrite (Cabail and Pacheco, 2003). This study used a NO concentration about 400-fold lower than the Hendrich *et al*. study and produced NO by photolysis of added N,N′-bis(carboxymethyl)-N,N′-dinitroso-1,4-phenylenediamine. The authors did not observe a stable $\{FeNO\}^6$ complex and, although the data was not shown, their preliminary investigations indicated that higher concentrations of NO still led to the reduction of *c*-hemes of HAO, not oxidation. Given these two recent and contradictory studies, there is currently no resolution as to whether NO is indeed formed during NH_2OH oxidation. However, biochemical and physiological experiments clearly demonstrate that NO and N_2O are produced and released during ammonia oxidation.

Cytochrome P460 and Nitrogen Oxide Metabolism

The production of NO and N_2O during aerobic metabolism of ammonia is an important physiological and environmental issue. Besides the possible production of these gases from HAO activity, fewer well characterized enzymes could also potentially form NO and N_2O during ammonia oxidation. A separate P460 enzyme, encoded by the *cyp* gene, is maintained and expressed by *N. europaea* and the methanotroph, *Methylococcus capsulatus* Bath (Erickson and Hooper, 1972; Bergmann *et al*., 1998). Cytochrome P460 purified from *M. capsulatus* Bath was shown to catalyze the oxidation of NH_2OH to nitrite and is thought to be the HAO analogue that in combination with pMMO completes the oxidation of ammonia to nitrite. It is also likely, but not certain, that cytochrome P460 is expressed in *Methylosinus trichosporium* OB3b and *Methylocystis parvus* OBBP, as suggested by their oxidation of ammonia to nitrite and the appearance of a *cyp* gene homologue revealed by Southern blots (Bergmann *et al*., 1998). However, a cytochrome P460 enzyme has not yet been isolated from either of these methanotrophic strains. Additionally, cytochrome P460 has not been found in Type I methanotrophs even though *Methylomonas agile* produces N_2O and nitrite during growth on methane and ammonia, indicating that it does possess NH_2OH oxidizing activity (Krämer *et al*., 1990). Another interesting note is the discovery of two possible cytochrome P460 homologues in the genome of *Bradyrhizhobium japonicum*, implying that this enzyme may not be limited to AOB or methanotrophs (Kaneko *et al*., 2002).

Recently, a wild-type and mutated form of P460 from *N. europaea* was expressed in *Pseudomonas aeruginosa* to determine the role of the lysine crosslink to heme (as opposed to the tyrosine crosslink of P460 in HAO), the unique feature differentiating P460 from other c-type cytochromes (Bergmann and Hooper, 2003). Interestingly, removal of the lysine-heme crosslink resulted in the loss of catalytic activity, although substrate could still bind. The optical spectrum of the altered P460 closely resembled *c*' cytochromes, enzymes with strong evolutionary linkages to P460 cytochromes (Bergmann *et al*., 2000). For example, a cytochrome *c*' purified from *Methylococcus capsulatus* Bath shared many biochemical similarities to cytochrome *c*' from other bacteria; however, its amino acid sequence was much more similar to P460 cytochromes than to other *c*' cytochromes (Zahn *et al*., 1996a). Generally, cytochrome *c*' is a subclass of periplasmic heme-*c* containing polypeptides that catalyze one-electron transfer reactions. These cytochromes are found in many types of bacteria including sulfur oxidizers (Schmidt and DiSpirito, 1990), photosynthesizers (Meyer and Kamen, 1982; Monkara *et al*., 1993), pathogens (Tettelin *et al*., 2000), and denitrifiers (Suzuki *et al*., 1988; Gilmour *et al*., 1991). Cytochrome *c*' has been implicated in detoxification of NO to N_2O in multiple organisms and operates both aerobically and anaerobically (Moir, 1999; Cross *et al*., 2000; Cross *et al*., 2001; Anjum *et al*., 2002). Thus, detoxification of NO appears to be a necessary component of both inorganic nitrogen metabolism, *i.e*., nitrification and denitrification, and pathogenesis, *i.e*., evasion of host production of NO within macrophages. Given the biochemical and genetic similarities between cytochrome P460 and cytochrome *c*' in *N. europaea* and *M. capsulatus* Bath, the function of P460 in NO metabolism remains an interesting subject for investigation.

METABOLISM OF NITROGEN OXIDES

Significance

Although the majority of studies on inorganic nitrogen metabolism by ammonia oxidizers and equivalent functions in methanotrophs have focused mainly on the ammonia oxidation pathway, these organisms also exhibit a rich diversity of nitrogen oxide metabolism. These metabolic processes, including reduction of nitrite to NO and N_2O (known as aerobic or nitrifier denitrification), oxidation of NH_2OH to NO and N_2O (as discussed above), and reduction of NO_2 to N_2, have been the subject of several review articles (Jetten *et al*., 1997; Colliver and Stephenson, 2000; Wrage *et al*., 2001). It should be noted that levels of NO and N_2O produced by ammonia oxidizers are several orders of magnitude (ca. 10^3–10^6) lower than levels of nitrite produced during ammonia oxidation. Although NO and N_2O are minor products of ammonia oxidizer metabolism, they have very strong environmental effects. For example, over the past few years, the production of N_2O from aerobic and O_2-limited (rather than solely anaerobic) environments has been recognized as a significant contributor of N_2O release to the atmosphere, accelerating both global warming and destruction of the stratospheric ozone layer (Stein and Yung, 2003). Ammonia oxidizers, methanotrophs, and heterotrophic nitrifiers are thought to be dominant players that release N_2O from aerobic to microaerobic environments, including terrestrial (Webster and Hopkins, 1996; Gödde and Conrad, 1999; Pérez *et al*., 2001), marine (Dore *et al*., 1998; Naqvi *et al*., 1998), and managed ecosystems such as wastewater and landfills (Sümer *et al*., 1995; Itokawa *et al*., 1996; Mandernack *et al*., 2000).

Of the processes involving nitrogen oxide metabolism, the microaerobic to anaerobic reduction of nitrite to NO and N_2O has received the most attention. The benefits of nitrite reduction to ammonia oxidizing bacteria remain unknown, but three main hypotheses have been posited: (1) decreased competition for O_2 by removing the substrate for nitrite oxidizers (Poth and Focht, 1985); (2) energy conservation and production under low O_2 tensions (Abeliovich and Vonshak, 1992; Miller *et al*., 1993); and (3) detoxification of intermediates and products of nitrogen metabolism, *i.e*., NO, NH_2OH, and nitrite (Stein and Arp, 1998; Beaumont *et al*., 2002a). Generally, ammonia oxidizers produce more NO and N_2O from nitrite reduction than methane oxidizers, which is not surprising given differences in their primary energy-generating metabolism. However, significant levels of nitrite reduction to NO and N_2O, as well as NO consumption, have been measured from several strains of methanotrophs (Yoshinari, 1984; Krämer *et al*., 1990; Ren *et al*., 2000; Stein, 2001).

As with many processes in inorganic nitrogen metabolism, production of NO and N_2O by ammonia and methane oxidizers does not occur solely by the reduction of nitrite. Both groups of bacteria produce N_2O during ammonia oxidation, and ammonia oxidizers also produce NO (Hynes and Knowles, 1984; Krämer *et al*., 1990; Beaumont *et al*., 2002a; Sutka *et al*., 2003). As discussed above, the catalytic cycle of HAO in ammonia oxidizers, and presumably P460 in methane oxidizers, may involve a NO intermediate. Interestingly, a study of the isotopic composition of N_2O produced during NH_2OH oxidation by *N. europaea* and *M. capsulatus* Bath showed strong differences in site preference of ^{15}N in N_2O molecules (Sutka *et al*., 2003). This indicates that NH_2OH oxidizing enzymes among ammonia- and methane oxidizing strains have different catalytic properties, which is expected since HAO and P460 are structurally unique from each other. These different isotopic signatures of N_2O are also useful in distinguishing sources of N_2O produced in different environments (Mandernack *et al*., 2000; Pérez *et al*., 2001; Stein and Yung, 2003).

Candidate Genes for the Production of NO and N_2O

The reduction of nitrite to NO and N_2O by classical denitrifying bacteria generally requires the activity of two separate enzymes: nitrite reductase (NirK or NirS) and nitric oxide reductase (NorB) (Ferguson, 1998; Mandernack *et al*., 2000; Pérez *et al*., 2001; Stein and Yung, 2003). The genome sequence of *N. europaea* revealed a gene with similarity to *nirK*, the copper containing nitrite reductase of denitrifiers (Chain *et al*., 2003). This *nirK*-like gene in *N. europaea* is most similar to the *aniA* nitrite reductase gene from *Neisseria gonorrhoeae* (Mellies *et al*., 1997). The similarity of the *N. europaea* gene to *aniA* is in contrast to marine ammonia oxidizers that encode *nirK* genes most closely related to classical denitrifying bacteria, such as *Alcaligenes* spp. (Casciotti and Ward, 2001). Additionally, Southern blot experiments using a portion of the *nirK* gene from *Pseudomonas* sp. G-179 as a probe revealed potential *nirK* genes in the terrestrial

ammonia oxidizing species, *Nitrosolobus* sp. 24-C and *Nitrosospira* sp. NpAV (Bruns *et al*., 1998). In methanotrophs, genes with similarity to both *nirK* and *nirS* have been identified in the genome of *Methylomonas* sp. 16A, the first report of an organism maintaining both the copper and cytochrome-cd_1 nitrite reductases (Ye and Thomas, 2001). However, it remains unknown how nitrite reductase enzymes contribute to metabolic processes of this or any other methanotrophic strain.

The *N. europaea nirK* homologue is located at the end of a four-gene cluster containing a blue copper oxidase (*bco*), a monoheme cytochrome *c*, and a diheme cytochrome *c* (Chain *et al*., 2003). With the discovery of *nirK* genes in multiple ammonia oxidizing species, it has often been assumed that NirK enzymes are responsible for the bulk of nitrite reduction by all AOB. However, mutational analysis of the *nirK* gene in *N. europaea* suggested that it performs mainly in aerobic rather than anaerobic metabolism (Beaumont *et al*., 2002a; Stein, 2003). For example, under aerobic conditions the *nirK* mutant strain produced more N_2O than the wild-type strain and was more sensitive to toxic effects of nitrite (Stein and Arp, 1998; Beaumont *et al*., 2002a). Also, the ability of the *nirK* mutant to oxidize NH_2OH to nitrite was substantially hindered relative to the wild-type strain (Stein, 2003). Under microaerobic conditions, the *nirK* mutant strain was able to reduce nitrite to N_2O at the same rate as the wild-type strain using either NH_2OH or N_2H_4 as a source of reductant (Stein, 2003). Thus, at least in *N. europaea*, NirK does not appear to be directly responsible for microaerobic nitrite reduction. On a purely speculative note, perhaps NirK is an integral component of the ammonia oxidation pathway that protects enzymes from the toxic effects of nitrite.

Genes for the reduction of NO to N_2O have also been identified in several ammonia-oxidizing species (Casciotti and Ward, 2002; Chain *et al*., 2003). In *N. europaea* the nitric oxide reductase genes are organized in an operon, *norCBQD*, similar to that found in denitrifying bacteria such as *Pseudomonas* sp. G-179 (Chain *et al*., 2003). In contrast, *Methylomonas* sp. 16a encodes the cytochrome *b* nitric oxide reductase (qNOR) which derives electrons from quinol rather than *c*-type cytochromes (Hendriks *et al*., 2000; Ye and Thomas, 2001). This nitric oxide reductase is not linked to a bc_1-complex and is commonly found in nondenitrifying bacteria, presumably for detoxification of NO (Richardson, 2000; Büsch *et al*., 2002). As an exception, *Ralstonia eutropha* uses qNOR for denitrification (Cramm *et al*., 1999).

A membrane-bound protein fraction isolated from a *norB* mutant of *N. europaea* was incapable of reducing NO under anaerobic conditions, indicating that NorB does indeed perform as a nitric oxide reductase (Beaumont *et al*., 2002). However, expression of *norB* in wild-type *N. europaea* cells was not inhibited in the presence of O_2, unlike similar genes found in classical denitrifying species (Arai *et al*., 1999). The *norB* mutant cells of *N. europaea* had similar growth kinetics as wild-type cells and were not affected by lowered O_2 concentrations or elevated nitrite concentrations (Beaumont *et al*., 2002b). Furthermore, the *norB*-deficient cells produced the same amounts of NO and N_2O as the wild-type during aerobic growth. The only observable phenotype of *norB* mutant cells was a slowed rate of growth in the presence of sodium nitroprusside, a NO-producing chemical, suggesting that NorB is involved in detoxifying NO-produced during ammonia oxidation. Together, these observations indicate that the *nirK* and *norB* genes in *N. europaea* are not necessary for cells to catalyze nitrifier denitrification. Several biochemical and whole-cell studies of *N. europaea* have shown the potential for multiple enzymes to reduce nitrite to NO and N_2O under a variety of experimental conditions. Perhaps, then, there are uncharacterized enzymes yet to be found in ammonia oxidizers that actually catalyze the majority of nitrifier denitrification.

Biochemical Studies of NO and N_2O Production

Production of NO and N_2O from both aerobic and anaerobic pathways in *N. europaea* was initially documented in the 1960s (Falcone *et al*., 1963; Anderson, 1964; Hooper, 1968). Several properties of NO- and N_2O-producing enzymes extracted from cells of *N. europaea* are summarized in Table 3. The aerobic pathway of NO and N_2O production derives directly from NH_2OH oxidation as discussed above (Hooper and Terry, 1979). The anaerobic pathway generally involves the reduction of nitrite, and at least two distinct polypeptides have been described that catalyze the reaction—the blue copper cytochrome *c* oxidase (encoded by *bco* within the *nirK* gene cluster) (Miller and Wood, 1983; DiSpirito *et al*., 1985; Miller and Nicholas, 1985) and an unidentified copper-containing enzyme (Ritchie and Nicholas, 1974). Studies of partially purified proteins revealed intimate connections between hydroxylamine oxidase, nitrite reductase, and cytochrome *c* oxidase activities that

TABLE 3
Properties of Cellular Extracts and Purified Polypeptides from *Nitrosomonas europaea* That Produce NO and/or N_2O

Reference	Purity[a]	$+O_2^b$	MW (×1000)	Cu	Heme	Abs. Max[c] (oxidized)	Nitrite Reductase	Cytochrome *c* Oxidase	NH_2OH Oxidase
Falcone *et al*., 1963	Partial	No							Yes
Anderson, 1964	Partial	No							Yes
Hooper, 1968	Partial	Yes					Yes	Yes	Yes
Ritchie and Nicholas, 1972	Partial	Yes/no[d]			Yes		Yes	No	Yes
Ritchie and Nicholas, 1974[e]	Pure	No			No	590	Yes		No
Ritchie and Nicholas, 1974	Partial	No			Yes		Yes		Yes
Hooper and Terry, 1979	Pure	Yes			Yes		No	No	Yes
Miller and Wood, 1983[f]	Pure	No	120/35	Yes	No	598	Yes	Yes	
Miller and Nicholas, 1985[f]									
DiSpirito *et al*., 1985	Pure	No	127.5/40.1	Yes	No	607	Yes	Yes	

[a]Represents partial purifications of multiple polypeptides or polypeptides purified to homogeneity.
[b]Refers to whether the production of NO and/or N_2O required the presence of oxygen.
[c]Absorbance maximum of oxidized polypeptide.
[d]NO andN_2O were produced by protein fractions both aerobically and anaerobically.
[e]Two nitrite reductase enzymes were analyzed in this study—a purified enzyme and a hydroxylamine oxidase associated enzyme.
[f]Two studies, Miller and Wood and Miller and Nicholas, were conducted on this polypeptide. The combined information is represented.

were initially difficult to separate from one another (Hooper, 1968; Ritchie and Nicholas, 1972). The connections among these activities are interesting given the slowed NH_2OH oxidation rate of the *nirK* mutant, suggesting that this particular nitrite reductase is involved in the smooth functioning of HAO catalysis and not in the microaerobic reduction of nitrite. Eventually, the copurified enzymes were separated from one another using sucrose gradients in combination with other methods, but the blue copper oxidase still maintained both cytochrome *c* oxidase and nitrite-reductase activities (Table 3). Unfortunately, with the exceptions of HAO, blue copper oxidase, and NirK, no other candidate genes have been identified in the genome of *N. europaea* with similarity to characterized nitrite-reducing enzymes. Thus, the remaining nitrite reducing enzymes will have to be revealed using modern physiological and/or genetic techniques as discussed below.

Physiology of Nitrogen Oxide Metabolism

Besides the mystery of genes and enzymes involved, it is also unknown why ammonia oxidizers catalyze nitrite reduction and why the process is enhanced as O_2 levels decrease. The most straightforward reason is to acquire energy under O_2-limited conditions. To date, two studies have demonstrated that cells of *N. europaea* can fix CO_2 into cellular material under anaerobic conditions via nitrite reduction, but only in the presence of ammonia (Abeliovich and Vonshak, 1992; Hyman and Arp, 1995). The only energy sources that could drive CO_2 fixation under anaerobic conditions were pyruvate and hydrazine. Interestingly, NH_2OH, other nitrogen sources, and substrates of AMO could not provide energy for CO_2 fixation under nitrite-reducing conditions. The requirement for ammonia under anaerobic conditions was considered as a mandatory

source of nitrogen and/or as a transcriptional activator rather than an energy source (Hyman and Arp, 1995; Sayavedra-Soto *et al*., 1996).

The most successful cultivated ammonia oxidizer in terms of anaerobic metabolism and growth is *N. eutropha*. Unlike other cultivated strains, *N. eutropha* appears to have a second type of ammonia monooxygenase activity (as described above) that oxidizes ammonia anaerobically to generate energy (Schmidt *et al*., 2001). This process requires NO_2 as the oxidant and produces NO (Zart *et al*., 2000). Although nitrite is not a necessary substrate for anaerobic metabolism, it can be used as an oxidant with H_2 as an energy source with the concomitant production of both N_2 and N_2O (Bock, 1995). No other ammonia-oxidizing strain has demonstrated similar versatility in anaerobic metabolism.

The majority of physiological studies of nitrite reduction under microaerobic or anaerobic conditions by whole cells of ammonia oxidizers have failed to demonstrate energy production or other direct benefits to the cells. These studies are both numerous and in general agreement with one another: nitrite reduction is greatly stimulated as O_2 levels decrease, and the process creates both NO and N_2O (Ritchie and Nicholas, 1972; Goreau *et al*., 1980; Lipschultz *et al*., 1981; Hynes and Knowles, 1984; Poth and Focht, 1985; Anderson and Levine, 1986; Remde and Conrad, 1990; Anderson *et al*., 1993; Kester *et al*., 1997; Dundee and Hopkins, 2001). The range of N_2O production reported in these studies is summarized in Table 4. Methanotrophs, namely *Methylosinus trichosporium* OB3b and *Methylomicrobium album* BG8, also produce N_2O microaerobically in the presence of ammonia and methane, or nitrite and reductant (Yoshinari, 1984; Ren *et al*., 2000; Stein, 2001). As stated above, the genes and enzymes that carry out this process are unknown for both ammonia and methane oxidizers, thus it is also unknown

TABLE 4
Rates of N_2O Production Under Microaerobic to Anaerobic Conditions by Ammonia and Methane Oxidizers

Organism	Substrate	Rate[a]	$\%O_2$	pH	References
Ammonia Oxidizers					
Marine *Nitrosomonas* sp.	24 mM NH_4^+	2,100	0.5	7.5	Goreau *et al*., 1980
Nitrosovibrio sp.	10 mM NH_4^+	34,000	20 to 0	7.4	Remde and Conrad, 1990
Nitrosomonas europaea	10 mM NH_4^+	330,000	20 to 0	7.4	Remde and Conrad, 1990
N. europaea	2 mM NH_4^+, 1 mM NO_2^-	3.0	0.0	7.5	Hynes and Knowles, 1984
N. europaea	15 mM NH_4^+	24	5.0	8.2	Anderson, Poth *et al*., 1993
N. europaea	20 mM NO_2^-	8.5	0.0	7.5	Ritchie and Nicholas, 1972
N. europaea	50 mM NH_4^+, 20 mM NO_2^-	8.2	0.0	7.5	Ritchie and Nicholas, 1972
N. europaea	0.5 mM NH_2OH, 5 mM NO_2^-	165	1.0	7.5	Stein, 2001
N. europaea	4 mM NH_4^+	6,700	5.0	7.0	Dundee and Hopkins, 2001
Nitrosolobus multiformis	4 mM NH_4^+	3,300	5.0	7.0	Dundee and Hopkins, 2001
Methanotrophs					
Methylosinus trichosporium	10 mM NH_4^+, 10% CH_4	1.5	3.7	6.8	Yoshinari, 1984
Methylomicrobium album	0.5 mM NH_2OH, 5 mM NO_2^-	7.0	1.0	6.5	Stein, 2001

[a]nmol $N_2O \cdot 10^9 cells^{-1} \cdot hr^{-1}$. Rates are the highest reported in each study. Other studies of N_2O production by these bacteria that did not report cell number are not included.

TABLE 5
Summary of Inorganic Compounds Associated with Metabolism of Ammonia-Oxidizing Bacteria

Compound	Structure	Role in metabolism
Ammonia/Ammonium	NH_3/NH_4^+	Electron donor, N source
Nitrite	NO_2^-	Product of ammonia oxidation, substrate for denitrification
Hydroxylamine	NH_2OH	Intermediate in the oxidation of ammonia to nitrite
Nitrous oxide	N_2O	Denitrification product, HAO side reaction
Nitric oxide	NO	Denitrification product, HAO side reaction, product of anoxic ammonia oxidation
Nitrogen dioxide/dinitrogen tetraoxide	NO_2/N_2O_4	Alternative oxidant for ammonia oxidation

whether there are evolutionary connections among the pathways in these two bacterial groups as for ammonia oxidation.

Although the majority of studies support the successive enzymatic reduction of nitrite to NO to N_2O, a question remains of how much chemodenitrification contributes to the production of these gases from abiological reactions of ammonia, NH_2OH, and nitrite (van Cleemput and Baert, 1984). In one study, fully aerobic cultures of *N. europaea* lost up to 8% inorganic nitrogen from a chemical interaction of NH_2OH and nitrite to produce N_2O (Stüven *et al.*, 1992). However, two studies using ^{15}N-labeled substrates clearly demonstrated that nitrite reduction was the dominant mechanism for N_2O production via an enzymatic process under microaerobic conditions (Ritchie and Nicholas, 1972; Poth and Focht, 1985). Likely, both biological and abiological reactions occur simultaneously to transform inorganic nitrogen compounds into NO and N_2O. However, the majority of N_2O production does appear to be enzymatically driven.

Besides the production of NO and N_2O, a *Nitrosomonas* sp. also produced N_2 during nitrite reduction, but this trait is generally rare among cultivated isolates (Poth, 1986). As an exception, one study showed up to 7% conversion of ammonium to N_2 by cells of *N. europaea* (ATCC, 19718) during successive transfer of a culture from aerobic to anaerobic conditions (Shrestha *et al.*, 2002). Unfortunately, this study lacked a killed cell control, did not show replicated treatments, and necessarily invoked the activity of a nitrous oxide reductase, which is not present in the genome of this strain (Chain *et al.*, 2003). Furthermore, no other researchers have demonstrated N_2 production by this strain. Thus, it is still largely accepted that nitrifier denitrification by *N. europaea* ends with the production of N_2O, but the universality of this pathway among ammonia oxidizing species remains unexplored.

CONCLUSIONS

Our knowledge of and appreciation for the roles of inorganic N compounds in the metabolism of AOB continue to grow (Table 5). From quantitative and physiological standpoints, the most significant of these compounds are ammonia and nitrite as the predominant substrate and product, respectively, of the AOB. All of the ammonia oxidized to nitrite will pass through the intermediate, hydroxylamine. However, this intermediate is not released to the environment. Two gaseous N oxides, NO and N_2O, are produced in smaller amounts relative to the amount of ammonia oxidized, but their release to the atmosphere has significant consequences. Trace amounts of N_2 have also been observed. Some AOB are capable of using NO_2 in addition to O_2 as an oxidant for the oxidation of NH_3. Nitrite is also a substrate for both oxic and anoxic denitrification by AOB.

Obviously, many questions remain to be answered regarding inorganic nitrogen metabolism in both ammonia- and methane-oxidizing bacteria. Fortunately, with closure of the *N. europaea* genome and in anticipation of complete genome sequences of other ammonia and methane oxidizers, the field is positioned to use postgenomic tools to uncover and link metabolic pathways as never before. For example, a genomic microarray of the *N. europaea* genome has been created and is currently being used to look at global gene expression of cells incubated under starvation-versus-growth conditions (Yan *et al.*, 2003). This array can also be used to examine differences in gene expression in mutant versus wild-type strains of *N. europaea*, or to find

similarities among gene structure and expression in related bacterial species.

The most significant contributions to be made for understanding inorganic nitrogen metabolism by ammonia oxidizers are to: (1) demonstrate the universality of genes and activities in multiple ammonia-oxidizing strains, (2) uncover all of the genes involved in major metabolic pathways, (3) show linkages between aerobic and microaerobic pathways for gaseous NO and N_2O production, and (4) demonstrate evolutionary conservation among pathways in ammonia and methane oxidizers. The roles of HAO and P460 are especially intriguing in regard to linking aerobic to microaerobic metabolism, and ammonia to methane-oxidizing bacteria. Hopefully, postgenomic tools will allow us to tease apart the components of each of these pathways and to establish the connections among the pathways. Within a few years and with the completion of more bacterial genome sequences, a new suite of tools will be available to finally answer long-held questions regarding inorganic nitrogen metabolism by both ammonia and methane oxidizing bacteria.

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