Regulation of the Synthesis and Activity of Ammonia Monoxygenase in *Nitrosomonas europaea* by Altering pH To Affect NH₃ Availability

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The obligately ammonia-oxidizing bacterium *Nitrosomonas europaea* was incubated in medium containing 50 mM ammonium. Changes in the concentration of nitrite, the pH, and the NH₄⁺- and NH₂OH-dependent O₂ uptake activities of the cell suspension were monitored. The NH₄⁺-dependent O₂ uptake activity doubled over the first 3 h of incubation and then slowly returned to its original level over the following 5 h. The extent of stimulation of NH₄⁺-dependent O₂ uptake activity was decreased by lowering the initial pH of the medium. Radiolabeling studies demonstrated that the stimulation of NH₄⁺-dependent O₂ uptake activity involved de novo synthesis of several polypeptides. Under O₂-limited conditions, the stimulated NH₄⁺-dependent O₂ uptake activity was stabilized. Rapid, controlled, and predictable changes in this activity could be caused by acidification of the medium in the absence of ammonia oxidation. These results indicate that the NH₄⁺-dependent O₂ uptake activity in *N. europaea* is strongly regulated in response to NH₄ concentration.

*Nitrosomonas europaea*, an obligate ammonia oxidizer, has been used extensively as a model organism due to the ease of its cultivation relative to other obligately ammonia-oxidizing bacteria (9). The energy-generating metabolism of *N. europaea*, the oxidation of ammonia to nitrite (NO₂⁻), is catalyzed by two enzymes. The first enzyme, ammonia monooxygenase (AMO), oxidizes ammonia to hydroxylamine (NH₂OH) and consumes molecular oxygen and two electrons in the process (13). The second enzyme in the ammonia oxidation pathway, hydroxylamine oxidoreductase (HAO), oxidizes NH₂OH to NO₂⁻, releasing four electrons. Two of these electrons are consumed by AMO to sustain further ammonia oxidation, and two are diverted either through the electron transport chain for ATP generation or to reversed electron flow for reduction of nucleotide cofactors used for CO₂ fixation and other biosynthetic reactions (13).

External pH is known to greatly influence the distribution and activity of ammonia-oxidizing bacteria in the environment (9). Part of this effect is thought to be due to the influence of pH on NH₃ availability (1, 12). The physiological substrate for AMO is NH₃, not NH₄⁺ (12). NH₃ is a curious substrate because at physiologically relevant pH values (pH 6 to 8), the NH₃-NH₂OH equilibrium is shifted primarily toward NH₂OH (pKa = 9.1 at 30°C) (11). Although autotrophic ammonia-oxidizing bacteria are not thought to be particularly active in acid soils because of limited NH₃ availability, these organisms can often be isolated from acidic environments (4). In contrast to the effects of NH₃ on AMO activity, we have recently demonstrated that the synthesis of the polypeptides required for ammonia-oxidizing activity in *N. europaea* is also regulated by NH₄⁺, not NH₃ (13).

In the present study we investigated the regulatory roles of NH₃ and NH₄⁺ on ammonia-oxidizing activity in cells of *N. europaea* with active rather than acetylene-inactivated AMO.

**Cell growth and analysis of batch incubations.** Cells of *N. europaea* ATCC 19178 were grown in 1.5-liter batches on rotary shakers (200 rpm) in 2-liter Erlenmeyer flasks at 30°C in the dark. Cells were harvested, washed, and stored overnight as described previously (5). Batch incubation mixtures containing growth medium (25 ml; 50 mM NH₄⁺, 50 mM sodium and potassium phosphate, trace minerals [5]) and washed *N. europaea* cells (250 μl) were incubated on a rotary shaker (200 rpm) in an unlit constant-temperature (30°C) room. At various times, samples (1 ml) of the reaction medium were removed, and the cells in these samples were sedimented by centrifugation (14,000 × g, 2 min). The resulting supernatant was collected and used to determine the pH of the reaction medium and the accumulation of nitrite as described previously (3). The cells were washed and used to measure the NH₄⁺- and NH₂OH-dependent O₂ uptake activities (5) and protein concentrations as described previously (2). Incubations with limiting O₂ were conducted in glass serum vials (160 ml) sealed with butyl rubber and aluminum crimp seals. The vials were evacuated and reequilibrated five times with O₂-free N₂. The desired amount of pure O₂ was added with a syringe. Incubation of cells with Na⁺14CO₃ or 14C₂H₄ and the separation of radiolabeled polypeptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were conducted as described previously (5). Incorporation of 14C into separate polypeptides was analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) by using Imagequant software (Molecular Dynamics). The P/2 program (Molecular Dynamics) was used to display labeled proteins in line graph...
that the effects on AMO activity were independent of cell growth. The peak and subsequent decline in AMO activity coincided with a sharp decrease in the rate of nitrite production (Fig. 1B). The concurrent decline in both AMO activity and the rate of nitrite production occurred even though only about 50% (25 mM) of the total ammonium had been oxidized (Fig. 1B). Both the pH and the calculated free \([\text{NH}_3]\) also declined significantly during the reaction (Fig. 1C).

At the point of maximal AMO activity, the pH had dropped from approximately 8.2 to 6.8 and the free \([\text{NH}_3]\) had dropped from 3.9 mM to approximately 47 \(\mu\)M. After 8 h, the pH had declined to 5.6, and only 6 \(\mu\)M \(\text{NH}_3\) remained.

**Effect of pH on AMO activity.** The results in Fig. 1 suggest that the observed changes in AMO activity correlated with the availability of \(\text{NH}_3\), rather than \(\text{NH}_4^+\). We therefore examined whether the increase in AMO activity over the first 4 h could be influenced by the initial concentration of available \(\text{NH}_3\) in the incubations.

Incubations were conducted by using phosphate (50 mM)-buffered media (pH 8) which contained 5, 15, or 50 mM ammonium. In all cases, the AMO activity underwent an initial rapid increase that was followed by a slower decrease similar to that shown in Fig. 1. The maximal level of AMO activity increased with increasing ammonium concentration (1.5-fold with 5 mM ammonium, 1.7-fold with 15 mM ammonium, and 2-fold with 50 mM ammonium) (data not shown). The strength of the buffer in the incubation medium also affected the time course of changes in AMO activity. Incubations were conducted with media containing 50 mM ammonium and various concentrations of sodium phosphate buffer (10 to 40 mM) at a constant starting pH of 8.1. Again, a rapid stimulation of AMO activity was observed in all incubations, which was followed by a slower decline to the original level of enzyme activity. However, decreases in buffering strength resulted in less maximal stimulation of AMO activity (1.5-fold with 10 mM phosphate, 1.6-fold with 20 mM phosphate, and 1.8-fold with 40 mM phosphate) and a shorter period over which maximal activity was observed (data not shown).

To further illustrate the effect of pH change on \(\text{NH}_3\) availability and the concurrent effect on AMO activity, batch incubations were conducted with medium containing 50 mM ammonium and sodium phosphate buffer (50 mM) adjusted to different initial pH values (pH 8.1, 7.5, and 6.3); the starting concentrations of free \(\text{NH}_3\) in these incubations were estimated to be 4.76, 1.43, and 0.08 mM, respectively. As the initial pH of the medium was decreased, there was also a decrease in the maximal stimulation of AMO activity (1.3-fold at pH 6.3, 1.7-fold at pH 7.5, and 2.2-fold at pH 8.1) (data not shown).

**Analysis of de novo polypeptide synthesis.** To determine if the increase in AMO activity was due to de novo protein synthesis, transcription or protein synthesis inhibitors were added to the incubation medium. Both rifampin and chloramphenicol greatly diminished the stimulation of AMO activity (Fig. 2). In contrast, HAO activity was not affected by the presence of either inhibitor. To determine which proteins were synthesized in response to \(\text{NH}_3\), \(\text{Na}_2^{14}\text{CO}_3\) was included with the cells to follow \(\text{14C}\) incorporation into newly synthesized polypeptides. In the presence of either chloramphenicol (Fig. 3A) or rifampin (Fig. 3B) there was no detectable incorporation of \(\text{14C}\) into polypeptides, indicating that there was a lack of de novo protein synthesis. In the absence of chloramphenicol or rifampin, several polypeptides had \(\text{14C}\) incorporated after 3 h (Fig. 3D). The four main labeled polypeptides corresponded to apparent molecular masses of 27, 42, 55, and 68 kDa. The highest level of \(\text{14C}\) was incorporated in the 27-kDa polypeptide. Between 3 and 8 h there was an approximately two-fold increase in the amounts of label associated with these polypeptides, indicating that there was a rapid stimulation of AMO activity was observed in all incubations, which was followed by a slower decline to the original level of enzyme activity. However, decreases in buffering strength resulted in less maximal stimulation of AMO activity (1.5-fold with 10 mM phosphate, 1.6-fold with 20 mM phosphate, and 1.8-fold with 40 mM phosphate) and a shorter period over which maximal activity was observed (data not shown).

**Changes in AMO and HAO activities in cells incubated in batch cultures.** When cells of *N. europaea* were incubated in medium containing 50 mM ammonium, the \(\text{NH}_3\)-dependent O\(_2\) uptake rate (AMO activity) increased by 1.8- to 2.6-fold between 3 and 4 h after the start of the incubation (Fig. 1A), after which the activity slowly declined to near the initial level. No further changes in the rate of \(\text{NH}_4^+\)-dependent O\(_2\) uptake were observed for at least an additional 48 h (data not shown). The observed changes in activity were specific for AMO as the rate of \(\text{NH}_4\text{-OH}\)-dependent O\(_2\) uptake (HAO activity) remained largely unchanged throughout the course of the incubation (Fig. 1A). The lack of change in HAO activity or protein content (data not shown) during these incubations suggests
after 3 h to limited incubations which exhibited stabilized activity in the absence of ammonia oxidation, HCl was added (Fig. 4B). The error bars indicate variations in four replicate experiments.

Effects of pH changes on AMO activity in O2-limited incubations. We wished to determine whether the changes in AMO activity could be manipulated in a predictable fashion in incubations in which the pH of the medium and the relative proportions of NH4+ and NH3 could be changed without concurrent ammonia oxidation. To limit the rate of ammonia oxidation and the extent of the associated pH change, cells were exposed to limiting amounts of O2 (5% of the gas headspace) during incubation (Fig. 4). In an incubation vial with limiting O2, the AMO activity increased over the first 3 h to a level only slightly lower than that observed in incubations with ambient levels of O2. However, in contrast to the incubation with ambient O2, the AMO activity in the O2-limited incubation remained stable and then slowly declined after 5 h. The rate of nitrite production was much slower in the incubations with limiting O2 than in incubations with ambient O2 levels (Fig. 4B).

To determine if a decline in pH alone could lower the AMO activity in the absence of ammonia oxidation, HCl was added after 3 h to O2-limited incubations which exhibited stabilized AMO activity (Fig. 4A). When 50 μl of HCl (10 N) was added, the pH dropped from 7.2 to 5.4, a value which is similar to the final pH observed in the incubation conducted with ambient O2. When 25 μl of HCl was added, the pH immediately decreased from 7.2 to 6.4, and it continued to decline slightly over the next few hours. An immediate decrease in the AMO activity was also observed in this incubation, compared with the slow decline in activity in the O2-limited vial without HCl. However, cells from both incubations had the same amount of AMO activity after 8 h. Similar pH adjustments were also made to the medium in incubations which began under anoxic conditions to test whether the acidification nonspecifically affected the cells rather than specifically affected AMO activity. Adjustment of the pH in the anoxic incubation from 8.0 to 6.4 had no effect on either AMO or HAO activity (Fig. 4A).

To test the possible consequences of the pH change on protein composition, we monitored the fate of newly synthesized polypeptides during the pH adjustments by including Na3[14]CO3 in the incubations (Fig. 4C). The newly synthesized 27-kDa component of AMO (AMOa) continued to accumulate in the O2-limited incubations with or without pH adjustment. However, at the end of the incubation, there was a greater level of radiolabel in the O2-limited incubation without pH adjustment than in the O2-limited incubation with pH adjustment. Likewise, there was a greater level of de novo protein synthesis overall in the incubation containing ambient O2 than in the O2-limited incubations. Furthermore, several unique polypeptides were synthesized in the incubation containing ambient O2 levels compared to the O2-limited incubations.

Taken together, the experiments described in this study demonstrate that the activity of AMO in N. europaea can undergo large changes over subgenerational periods of time when cells are incubated in batch cultures containing ammonium. It is important to note that for all of the activity measurements described here we used cells that were washed after they were removed from their incubation mixtures. This ap-
changes in AMO activity described in this study (Fig. 1) are consistent with a response of \textit{N. europaea} to the ambient concentrations of \( \text{NH}_3 \) rather than \( \text{NH}_4^+ \). Furthermore, our results indicate that there are two components to the response to \( \text{NH}_3 \), one that leads to an increase in AMO activity through de novo protein synthesis and a second counteracting response that decreases the activity of AMO to a defined level in a process which does not require the degradation of newly synthesized AMO polypeptides. These observations confirm and expand our previous observations of the responses of \textit{N. europaea} to \( \text{NH}_3 \) (6, 10).

The mechanism by which \textit{N. europaea} increases AMO activity in response to \( \text{NH}_3 \) concentrations appears to involve selective de novo protein synthesis, as suggested by the effects of transcription and translation inhibitors (Fig. 2) and our \( ^{14} \text{CO}_2 \)-labeling studies (Fig. 3). The four main polypeptides synthesized during the increase in AMO activity (Fig. 3) have molecular weights similar to those observed during the recovery of AMO activity in \textit{N. europaea} after inactivation by either \( \text{C}_2 \text{H}_2 \) or light (5). The 27-kDa polypeptide observed in the present study is very likely to be AMOa (8) based on its comigration with the \( ^{14} \text{C} \)-labeled polypeptide generated by exposure of the cells to \( ^{14} \text{C}_2 \text{H}_2 \) (Fig. 3). Likewise, the 42-kDa polypeptide is probably the AMOb (8) component of AMO, although it is clear that the level of \( ^{14} \text{C} \) incorporation into this polypeptide is substoichiometric with respect to the level observed in the 27-kDa polypeptide. Therefore, the two known components of AMO (AMOa and AMOb) can be differentially synthesized. This result is surprising as the \textit{amoA} and \textit{amoB} genes are cotranscribed (10) and AMOa and AMOb polypeptides copurify in apparently equimolar concentrations (8). Although the regulatory significance of this differential synthesis of AMO components remains to be determined, it is important to note that this effect has been consistently observed in our recent studies of AMO regulation (5, 6), either during recovery from acetylene or light inactivation (5) or during \( \text{NH}_2 \text{OH} \)-dependent de novo protein synthesis by cells continuously exposed to acetylene (6). In addition to these putative components of AMO, we also observed the synthesis of 68- and 55-kDa polypeptides which was also substoichiometric with respect to AMOa. The identity of these polypeptides remains unknown.

The factors controlling the decrease in AMO activity also appear to be determined by the availability of \( \text{NH}_3 \) rather than \( \text{NH}_4^+ \), despite the fact that approximately 50% of the added ammonium remained unoxidized in most experiments. Our results also demonstrate that a net decrease in AMO activity consistently became apparent when the free \( \text{NH}_3 \) concentration in the incubation medium had decreased to approximately 50 \( \mu \text{M} \) (Fig. 1). This value is very similar to the \( K_m \) for \( \text{NH}_3 \) as a substrate for AMO (12) and the \( K_m \) for \( \text{NH}_3 \) as a regulatory agent during de novo protein synthesis (6). Our present results therefore provide further evidence that \textit{N. europaea} is able to regulate the activity of AMO at physiologically and enzymatically relevant concentrations of \( \text{NH}_3 \). While the increases in AMO activity involve de novo protein synthesis, the decrease in AMO activity observed in pH-adjusted, \( \text{O}_2 \)-limited incubations does not appear to involve protein degradation (Fig. 4).

Our results demonstrated that pH adjustment had no effect on cells incubated under anoxic conditions. These results suggest that cells of \textit{N. europaea} may be able to support two types of AMO activity. One of these types appears to provide a basal level of enzyme activity which is largely insensitive to changes in the available \( \text{NH}_3 \) concentration. This is the activity which was observed at the start of each incubation and the level to which the cells returned after they underwent an initial stimulation of activity and a subsequent decline. The second type of

FIG. 4. Effects of pH adjustment on \( \text{NH}_4^+ \)-dependent \( \text{O}_2 \) uptake activity, \( ^{14} \text{Cr} \) radiolabel distribution in \textit{N. europaea} incubated with \( \text{NH}_4^+ \) in the presence of limiting \( \text{O}_2 \) concentrations. Cells of \textit{N. europaea} were incubated in medium containing 50 mM ammonium in the presence of either ambient (20%) or limiting (5%, vol/vol) \( \text{O}_2 \) concentrations. Acidification of the incubation medium with HCl was performed after 3 h where indicated. (A) \( \text{NH}_4^+ \)-dependent \( \text{O}_2 \) uptake rates for cells with ambient levels of \( \text{O}_2 \) (■), 5% \( \text{O}_2 \) (△), 5% \( \text{O}_2 \) and 25 \( \mu \text{l} \) of HCl (10 N) (▲), 5% \( \text{O}_2 \) and 50 \( \mu \text{l} \) of HCl (●), 0% \( \text{O}_2 \) (□), and 0% \( \text{O}_2 \) and 50 \( \mu \text{l} \) of HCl (●). The data for incubations performed with 5% \( \text{O}_2 \) in the headspace are given by heavy lines to distinguish the experimental incubations from the control incubations. (B) Concurrent \( \text{O}_2 \) production rates in incubations with ambient levels of \( \text{O}_2 \) (■), 5% \( \text{O}_2 \) (△), 5% \( \text{O}_2 \) and 25 \( \mu \text{l} \) of HCl (▲), 5% \( \text{O}_2 \) and 50 \( \mu \text{l} \) of HCl (●), 0% \( \text{O}_2 \) (□), and 0% \( \text{O}_2 \) and 50 \( \mu \text{l} \) of HCl (●). The data for incubations performed with 5% \( \text{O}_2 \) are given by heavy lines. (C) Cells were incubated in the presence of Na\(^{14} \text{CO}_3 \), and radiolabel incorporation into de novo-synthesized polypeptides was visualized by SDS-PAGE as described in the text. The Phosphorimage of the SDS-PAGE gel is shown for cells incubated for 0, 3, 4, or 8 h with 5% \( \text{O}_2 \), 5% \( \text{O}_2 \) plus 50 \( \mu \text{l} \) of HCl, and ambient \( \text{O}_2 \). The arrow indicates the position of the putative AMOa polypeptide at 27 kDa. Relative densitometric values for radiolabel incorporation into the 27-kDa polypeptide are shown for each lane.

proach ensured that all of the reported enzyme activities were determined under identical conditions and that any differences reflect the effects of the incubation conditions rather than the enzyme assay conditions. With this in mind, we suggest that the enzyme assay conditions. With this in mind, we suggest that the
AMO activity was represented and characterized in the present study. This activity can be increased in response to increases in NH₃ availability and can be rapidly decreased in response to NH₃ limitation. These two differentially regulated forms of enzyme activity could be particularly useful to *N. europaea* for a rapid response to transient fluctuations in ammonia availability and still allow the organism to maintain a basal level of AMO activity to generate energy for both cell maintenance and the rapid de novo synthesis of protein once ammonia becomes available. While the presence of two apparently different forms of AMO activity is consistent with our present results, we have not yet determined whether these forms are related to the two copies of the AMO structural genes present in the *N. europaea* genome (8).

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REFERENCES