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Regulation of the Synthesis and Activity of Ammonia Monooxygenase in *Nitrosomonas europaea* by Altering pH To Affect NH_3 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 NH_4^+- and NH_2OH-dependent O_2 uptake activities of the cell suspension were monitored. The NH_4^+-dependent O_2 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 NH_4^+-dependent O_2 uptake activity was decreased by lowering the initial pH of the medium. Radiolabeling studies demonstrated that the stimulation of NH_4^+-dependent O_2 uptake activity involved de novo synthesis of several polypeptides. Under O_2-limited conditions, the stimulated NH_4^+-dependent O_2 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 NH_4^+-dependent O_2 uptake activity in *N. europaea* is strongly regulated in response to NH_3 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 (NO_2^-), is catalyzed by two enzymes. The first enzyme, ammonia monooxygenase (AMO), oxidizes ammonia to hydroxylamine (NH_2OH) and consumes molecular oxygen and two electrons in the process (13). The second enzyme in the ammonia oxidation pathway, hydroxylamine oxidoreductase (HAO), oxidizes NH_2OH to NO_2^-, 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 CO_2 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 NH_3 availability (1, 12). The physiological substrate for AMO is NH_3, not NH_4^+ (12). NH_3 is a curious substrate because at physiologically relevant pH values (pH 6 to 8), the NH_3-NH_4^+ equilibrium is shifted primarily toward NH_4^+ (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 NH_3 availability, these organisms can often be isolated from acidic environments (4). In contrast to the effects of NH_3 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 NH_3, not NH_4^+. In one study, the regulatory effects of ammonia were investigated by following de novo protein synthesis in acetylene-inactivated cells which were unable to use ammonia oxidation as a source of energy (6). This study demonstrated that the de novo synthesis of the 27-kDa component of AMO is a saturable process with respect to $[NH_3]$ rather than $[NH_4^+]$ (K_s $[NH_3]$ = 30 μM) (6). Similar results were observed in a second study which examined the regulatory effects of ammonia on the transcription of AMO-encoding genes in both acetylene-inactivated and control cells (10). Taken together, these studies indicate that NH_3 affects the ammonia-oxidizing activity of *N. europaea* at both the transcriptional and translational levels.

In the present study we investigated the regulatory roles of NH_3 and NH_4^+ 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 NH_4^+, 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 NH_4^+- and NH_2OH-dependent O_2 uptake activities (5) and protein concentrations as described previously (2). Incubations with limiting O_2 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 O_2-free N_2. The desired amount of pure O_2 was added with a syringe. Incubation of cells with $Na_2{}^{14}CO_3$ or $^{14}C_2H_2$ and the separation of radiolabeled polypeptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were conducted as described previously (5). Incorporation of ^{14}C into separate polypeptides was analyzed with a Phosphorimager (Molecular Dynamics, Sunnyvale, Calif.) by using Imagequant software (Molecular Dynamics). The Peakfind program (Molecular Dynamics) was used to display labeled proteins in line graph

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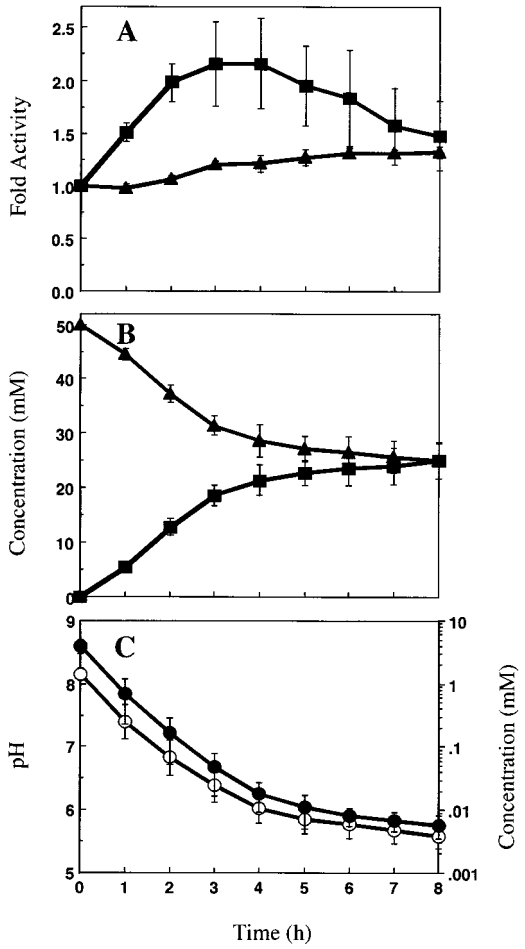

FIG. 1. Time course of changes in enzyme activities and culture conditions for cells of *N. europaea* incubated in medium containing 50 mM ammonium. Cells were incubated in medium containing 50 mM ammonium and sampled for enzyme activities as described in the text. The error bars indicate variations in four replicate experiments. (A) Time course for changes in NH_4^+-dependent (AMO) (■) and NH_2OH-dependent (HAO) (▲) O_2 uptake activities. (B) Time course of NO_2^- production (■) and estimated residual NH_4^+ concentration (▲). (C) Time course for changes in medium pH (○) and estimated concentration of NH_3 in solution (●), as determined from pH measurements and calculated residual NH_4^+ concentrations.

format based on the intensity of the labeled bands. The apparent molecular masses of the major polypeptides were determined by comparison with R_f values obtained for molecular mass markers. The markers used were bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), cytochrome *c* (12.5 kDa), and aprotinin (6 kDa) (Sigma Chemical Co.).

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 NH_4^+-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 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 NH_2OH-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 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 [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 [NH_3] had dropped from 3.9 mM to approximately 47 μM. After 8 h, the pH had declined to 5.6, and only 6 μM 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 NH_3 rather than 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 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 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 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 NH_3, $Na_2{}^{14}CO_3$ was included with the cells to follow ^{14}C incorporation into newly synthesized polypeptides. In the presence of either chloramphenicol (Fig. 3A) or rifampin (Fig. 3B) there was no detectable incorporation of ^{14}C into polypeptides, indicating that there was a lack of de novo protein synthesis. In the absence of chloramphenicol or rifampin, several polypeptides had ^{14}C 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 ^{14}C 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

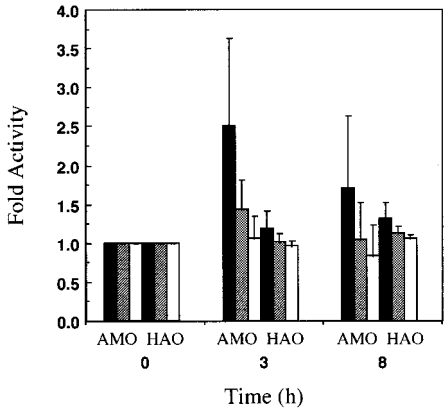

FIG. 2. Effect of RNA and protein synthesis inhibitors on changes in NH_4^+- and NH_2OH-dependent O_2 uptake rates. Cells of *N. europaea* were incubated in medium containing 50 mM ammonium and chloramphenicol (400 μg/ml) or rifampin (400 μg/ml). NH_4^+-dependent (AMO) and NH_2OH-dependent (HAO) O_2 uptake activities are shown for cells incubated for 0, 3, and 8 h with no inhibitor (solid bars), with chloramphenicol (shaded bars), and with rifampin (open bars). The error bars indicate variations in four replicate experiments.

with each major polypeptide (Fig. 3E). Thus, during the decline in AMO activity, the amount of labeled protein did not decrease; rather, it continued to accumulate. To determine which polypeptide corresponded to the 27-kDa component of AMO (AMOa), a separate aliquot of cells was incubated in the presence of $^{14}C_2H_2$ but without $Na_2{}^{14}CO_3$ (7). The ^{14}C-labeled polypeptide generated in this assay comigrated with the 27-kDa polypeptide labeled by fixation of $^{14}CO_2$ (Fig. 3F).

Effects of pH changes on AMO activity in O_2-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 NH_3 and NH_4^+ 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 O_2 (5% of the gas headspace) during incubation (Fig. 4). In an incubation vial with limiting O_2, the AMO activity increased over the first 3 h to a level only slightly lower than that observed in incubations with ambient levels of O_2. However, in contrast to the incubation with ambient O_2, the AMO activity in the O_2-limited incubation remained stable and then slowly declined after 5 h. The rate of nitrite production was much slower in the incubations with limiting O_2 than in incubations with ambient O_2 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 O_2-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 concentrations of O_2. Following the pH adjustment, there was a large, rapid decline in the AMO activity that approached the level observed in the incubation containing ambient O_2. 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 O_2-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 $Na_2{}^{14}CO_3$ in the incubations (Fig. 4C). The newly synthesized 27-kDa component of AMO (AMOa) continued to accumulate in the O_2-limited incubations with or without pH adjustment. However, at the end of the incubation, there was a greater level of radiolabel in the O_2-limited incubation without pH adjustment than in the O_2-limited incubation with pH adjustment. Likewise, there was a greater level of de novo protein synthesis overall in the incubation containing ambient O_2 than in the O_2-limited incubations. Furthermore, several unique polypeptides were synthesized in the incubation containing ambient O_2 levels compared to the O_2-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-

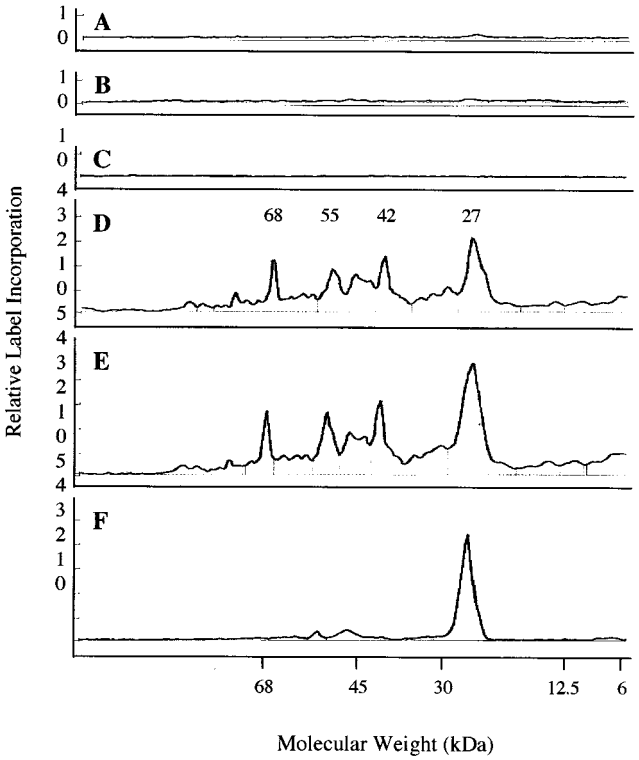

FIG. 3. Effects of inhibitors on the de novo synthesis of ^{14}C-labeled polypeptides. Cells of *N. europaea* were incubated in medium containing 50 mM ammonium with $Na_2{}^{14}CO_3$, and ^{14}C incorporation into newly synthesized polypeptides was analyzed by SDS-PAGE. The densitometric profiles of radiolabel distribution for cells incubated under the following conditions were determined: 8 h in medium containing chloramphenicol (400 μg/ml) (A); 8 h in medium containing rifampin (400 μg/ml) (B); 0 h in medium alone (C); 3 h in medium alone (D); 8 h in medium alone (E); and incubation in the absence of $Na_2{}^{14}CO_3$ for 3 h and subsequent exposure to $^{14}C_2H_2$ (F). The molecular masses were determined by comparing R_f values for individual polypeptides with the R_f values for a series of molecular mass markers as described in the text.

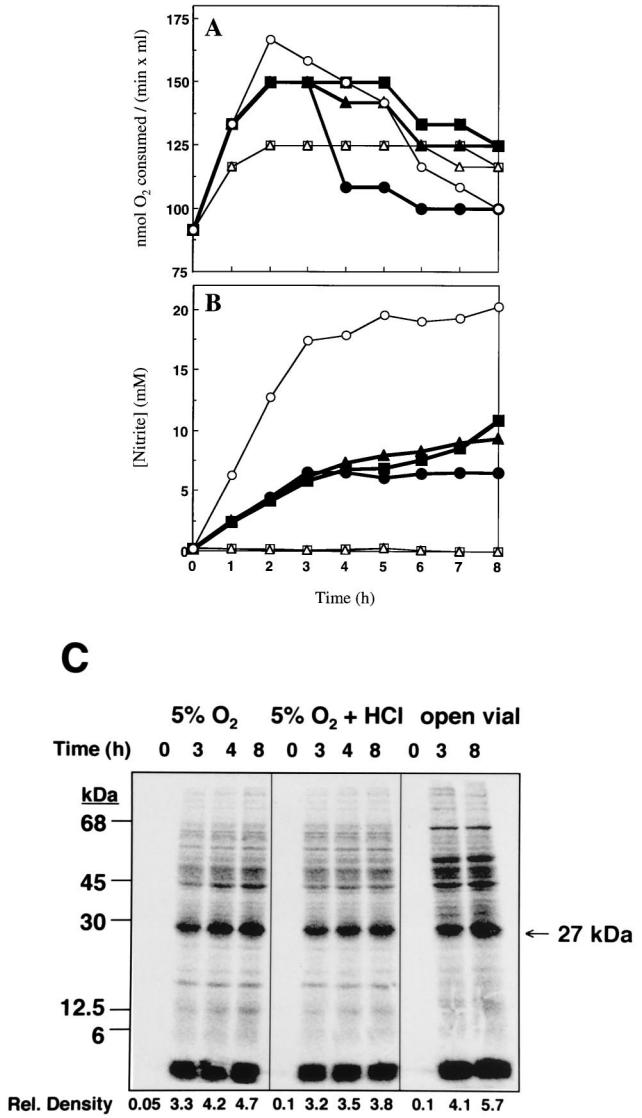

FIG. 4. Effects of pH adjustment on NH_4^+-dependent O_2 uptake activity, NO_2^- production, and ^{14}C radiolabel distribution in *N. europaea* incubated with NH_4^+ in the presence of limiting O_2 concentrations. Cells of *N. europaea* were incubated in medium containing 50 mM ammonium in the presence of either ambient (20%) or limiting (5%, vol/vol) O_2 concentrations. Acidification of the incubation medium with HCl was performed after 3 h where indicated. (A) NH_4^+-dependent O_2 uptake rates for cells with ambient levels of O_2 (○), 5% O_2 (■), 5% O_2 and 25 μl of HCl (10 N) (▲), 5% O_2 and 50 μl of HCl (●), 0% O_2 (□), and 0% O_2 and 50 μl of HCl (△). The data for incubations performed with 5% O_2 in the headspace are given by heavy lines to distinguish the experimental incubations from the control incubations. (B) Concurrent NO_2^- production rates in incubations with ambient levels of O_2 (○), 5% O_2 (■), 5% O_2 and 25 μl of HCl (▲), 5% O_2 and 50 μl of HCl (●), 0% O_2 (□), and 0% O_2 and 50 μl of HCl (△). The data for incubations performed with 5% O_2 are given by heavy lines. (C) Cells were incubated in the presence of $Na_2{}^{14}CO_3$, 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% O_2, 5% O_2 plus 50 μl of HCl, and ambient 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 changes in AMO activity described in this study (Fig. 1) are consistent with a response of *N. europaea* to the ambient concentrations of NH_3 rather than NH_4^+. Furthermore, our results indicate that there are two components to the response to 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 *N. europaea* to NH_3 (6, 10).

The mechanism by which *N. europaea* increases AMO activity in response to 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 $^{14}CO_2$-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 *N. europaea* after inactivation by either C_2H_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}C-labeled polypeptide generated by exposure of the cells to $^{14}C_2H_2$ (Fig. 3). Likewise, the 42-kDa polypeptide is probably the AMOb (8) component of AMO, although it is clear that the level of ^{14}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 *amoA* and *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 NH_2OH-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 NH_3 rather than 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 NH_3 concentration in the incubation medium had decreased to approximately 50 μM (Fig. 1). This value is very similar to the K_m for NH_3 as a substrate for AMO (12) and the K_s for NH_3 as a regulatory agent during de novo protein synthesis (6). Our present results therefore provide further evidence that *N. europaea* is able to regulate the activity of AMO at physiologically and enzymatically relevant concentrations of NH_3. While the increases in AMO activity involve de novo protein synthesis, the decrease in AMO activity observed in pH-adjusted, 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 *N. europaea* may be able to support two types of AMO activity. One of these types appears to provide a base level of enzyme activity which is largely insensitive to changes in the available 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

AMO activity was represented and characterized in the present study. This activity can be increased in response to increases in NH_3 availability and can be rapidly decreased in response to NH_3 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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