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Effects of Ammonia on the De Novo Synthesis of Polypeptides in Cells of *Nitrosomonas europaea* Denied Ammonia as an Energy Source

MICHAEL R. HYMAN* AND DANIEL J. ARP

The Laboratory for Nitrogen Fixation Research, Oregon State University, Corvallis, Oregon 97330

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The effects of ammonium on the de novo synthesis of polypeptides in the soil-nitrifying bacterium *Nitrosomonas europaea* have been investigated. Cells were incubated in the presence of both acetylene and NH_4^+. Under these conditions, the cells were unable to utilize NH_4^+ as an energy source. Energy to support protein synthesis was supplied by the oxidation of hydroxylamine or other alternative substrates for hydroxylamine oxidoreductase. De novo protein synthesis was detected by ^{14}C incorporation from $^{14}CO_2$ into polypeptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. In the presence of NH_4^+, acetylene-treated cells synthesized the 27-kDa polypeptide of ammonia monooxygenase (AMO) and two other major polypeptides (with sizes of 55 and 65 kDa). The synthesis of these polypeptides was completely inhibited by chloramphenicol and attenuated by rifampin. The optimal concentration of hydroxylamine for the in vivo ^{14}C-labeling reaction was found to be 2 mM. The effect of NH_4^+ concentration was also examined. It was shown to cause a saturable response with a K_s of approximately 2.0 mM NH_4^+. Labeling studies conducted at different pH values suggest cells respond to NH_3 rather than NH_4^+. No other compounds tested were able to influence the synthesis of the 27-kDa component of AMO, although we have also demonstrated that this polypeptide can be synthesized under anaerobic conditions in cells utilizing pyruvate- or hydrazine-dependent nitrite reduction as an energy source. We conclude that ammonia has a regulatory effect on the synthesis of a subunit of AMO in addition to providing nitrogen for protein synthesis.

Nitrosomonas europaea is an obligate lithotrophic bacterium which obtains all of its energy for growth from the aerobic oxidation of ammonia (NH_3) to nitrite (NO_2^-). This organism is also an autotroph, and the energy derived from ammonia oxidation is utilized in part to fix CO_2. Ammonia oxidation is initiated by the enzyme ammonia monooxygenase (AMO), which oxidizes ammonia to hydroxylamine (NH_2OH). As a monooxygenase, AMO utilizes NH_3 (28) and O_2 as substrates and requires two electrons to reduce one atom of oxygen to water, with the second oxygen atom being incorporated into ammonia, as follows: $NH_3 + O_2 + 2[H] \rightarrow NH_2OH + H_2O$. The resulting hydroxylamine is further oxidized to nitrite by the complex multiheme-containing enzyme, hydroxylamine oxidoreductase (HAO), as follows: $NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$. The four electrons (e) derived from this oxidative step provide the electrons required for both AMO activity and ATP synthesis (28).

There is considerable interest in the enzymology of ammonia-oxidizing nitrifying bacteria for several reasons. On the one hand, autotrophic nitrifying bacteria can have detrimental environmental effects. They are largely responsible for the often substantial losses of ammonium-based fertilizers used in agriculture. The nitrate generated by nitrification of fertilizer ammonia can represent a serious pollution problem in both surface water and groundwater. An effective means to reduce these losses of ammonia is to utilize nitrification inhibitors. A detailed understanding of the enzymology of ammonia oxidation could help considerably in the design of more effective nitrification inhibitors. On the more positive side, nitrifying bacteria are capable through the activity of AMO of oxidizing numerous hydrocarbon substrates including alkanes, alkenes, ethers, thioethers, aromatics, and halogenated hydrocarbons (12–16, 18, 19, 24, 27). The broad substrate range of AMO has raised the interesting possibility of utilizing autotrophic nitrifying bacteria for such beneficial purposes as bioremediation schemes.

Despite being such a versatile catalyst, AMO has not been purified in an active state. One of the most significant problems associated with the characterization of AMO is the large losses of activity which occur as a result of cell breakage. This effect is apparently partially due to the loss of copper from the enzyme. We have recently demonstrated that copper ions are unique in their ability to stimulate AMO activity in vitro (5). In contrast, recent progress on the molecular characterization of AMO has been derived more from catalytically inactive acetylene-treated enzyme rather than active enzyme preparations. The kinetics of the interaction of AMO and acetylene fulfill the kinetic criteria of mechanism-based inactivation (10, 17). Most notably, the use of $^{14}C_2H_2$ results in the covalent modification of a single membrane-bound 27-kDa polypeptide (10, 17) which is thought to contain the active site of AMO. Identification of this polypeptide has opened several avenues for further investigation. Most recently, the DNA sequence (*amoA*) encoding this polypeptide (AMOa) has been reported (22). A second gene (*amoB*) lies immediately downstream from *amoA* and encodes a 38-kDa polypeptide (AMOb). These polypeptides also copurify, and in combination, these observations have been taken to suggest that *amoA* and *amoB* encode structural genes of AMO (22). Little else is currently known about the molecular composition of this important enzyme.

The main aim of the experiments has been to investigate the effects of ammonia on protein synthesis in cells which have been denied ammonia as a source of energy. To investigate this

* Corresponding author. Mailing address: The Laboratory for Nitrogen Fixation Research, 2082 Cordley, Oregon State University, Corvallis, OR 97330. Phone: (503) 737-4214. Fax: (503) 737-3573. Electronic mail address: hymanm@bcc.orst.edu.

phenomenon, we have extended a previous technique which followed the recovery of ammonia-oxidizing activity in cells after the inactivation of preexisting AMO by acetylene (10). In these earlier experiments, we demonstrated that de novo protein synthesis is required to observe a recovery of ammonia-oxidizing activity. Radiolabeling techniques also demonstrated that the recovery of AMO activity was accompanied by the synthesis of a 27-kDa polypeptide (AMOa) and additional uncharacterized polypeptides (10). In the present study, we have followed de novo protein synthesis in acetylene-treated cells incubated under conditions in which ammonium is present as a potential inducer rather than as an energy source. To achieve this, we have employed the following steps. First, we inactivated preexisting AMO by incubating cells with acetylene. Subsequently, cells of *N. europaea* were prevented from utilizing ammonium as an energy source because all further incubations with ammonium were conducted in the continuous presence of acetylene. Under these circumstances, acetylene immediately and irreversibly inactivates any newly synthesized AMO. Accordingly, any newly synthesized protein is not able to contribute to the energy budget of the cell. The second step, which aims to provide an energy source in the absence of ammonia oxidation, made use of such HAO substrates as hydroxylamine and hydrazine to support de novo protein synthesis. The third step relied on the autotrophic nature of *N. europaea* and utilized $^{14}CO_2$ uptake in conjunction with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography to follow the incorporation of radiolabel into individual newly synthesized polypeptides.

MATERIALS AND METHODS

Materials. Cells of *N. europaea* (ATCC 19178) were grown and harvested as described previously (10). $Na_2{}^{14}CO_3$ (6.2 mCi/mmol) was supplied by Sigma Chemical Co. (St. Louis, Mo.). Electrophoresis reagents were supplied by Schwarz/Mann Biotech (Cleveland, Ohio). All other chemicals were of reagent grade.

Incubations with $Na_2{}^{14}CO_3$. Harvested cells were stored in buffer (20 mg of protein per ml in 50 mM sodium phosphate with 2 mM $MgCl_2$, pH 7.8) in the dark for between 3 and 24 h at 4°C. Before exposure to $Na_2{}^{14}CO_3$, the cells were treated with acetylene as follows to inactivate AMO activity. Cells (1 ml) were added to 20 ml of buffer in a glass serum vial (160 ml). The vial was stoppered with a butyl rubber stopper and aluminum crimp seal (Wheaton Scientific, Millville, N.J.). Acetylene was added to a final concentration of 1% (vol/vol) of the gas phase. The vial was incubated for 30 min on a rotary shaker (200 rpm) in a constant-temperature room (30°C). The cells were then removed from the vial and sedimented by centrifugation (10,000 × *g*, 10 min). The cells were resuspended in buffer (1 ml) and stored at 4°C. Control cells were treated exactly the same as described above, except that the treatment of the control cells took place in the absence of acetylene. Both acetylene-treated and control cells were added to incubations with $^{14}CO_2$ within 10 min of being resuspended in buffer.

Incubations of cells with $Na_2{}^{14}CO_3$ were conducted in glass serum vials (10 ml) stoppered with butyl rubber stoppers and aluminum crimp seals. Unless otherwise stated, each vial contained 5 μCi of $Na_2{}^{14}CO_3$ added from a solution of $Na_2{}^{14}CO_3$ in water (1 mCi/ml). This provided a 10-fold molar excess of $Na_2{}^{14}CO_3$ over unlabeled atmospheric CO_2 enclosed in the vial. Acetylene (1%, vol/vol) was added as a gas as an overpressure. All other additions to the vials were made from concentrated stock solutions in buffer. The balance of the aqueous reaction volume was then made up to 0.95 ml with buffer, and the sealed vials were equilibrated to 30°C in a shaking water bath. The reactions were initiated by the addition of the cells (50 μl, or approximately 1 mg of protein), and the vials were returned to the shaking water bath. The reactions were terminated after 30 min by removing 0.9 ml of the reaction mixture and centrifuging this in a 1.5-ml microcentrifuge tube (14,000 × *g* for 30 s). The supernatant was then carefully removed and stored separately while the pelleted cells were quickly resuspended in 2× SDS-PAGE sample buffer (200 μl) without β-mercaptoethanol. These protein samples were stored at −20°C until they were analyzed by SDS-PAGE and fluorography, as described below. Anaerobic incubations with $Na_2{}^{14}CO_3$ were conducted exactly as described above, except that all the solutions (except $Na_2{}^{14}CO_3$) and the reaction vial were made anaerobic by five cycles of evacuation and reequilibration with O_2-free N_2. The incubations were also extended to 2 h in duration.

Electrophoresis and fluorography. All protein samples were analyzed with a Protean II vertical slab gel electrophoresis apparatus (Bio-Rad Laboratories, Hercules, Calif.). Immediately prior to the loading of the samples onto SDS-PAGE gels, appropriate amounts of the protein samples (200 μg of protein) were vortexed and mixed with an equal volume of an aqueous solution of β-mercaptoethanol (10% [vol/vol]). A volume of this mixture containing 150 μg of protein was then loaded on the SDS-PAGE gel. All SDS-PAGE gels (1.5 mm by 16 by 14 cm) consisted of 13.5% (vol/vol) acrylamide resolving gels and 6% (vol/vol) stacking gels. All gels were electrophoresed at 4°C for 4 to 6 h with the discontinuous buffer system described by Laemmli (21). The gels were stained with Coomassie brilliant blue R-250 (0.1%, wt/vol) dissolved in an aqueous solution of acetic acid (7.5% vol/vol) and methanol (25%, vol/vol). The gels were destained in the same solution without dye. The apparent molecular masses of all the polypeptides were determined by comparison with the R_f values for molecular mass markers which were included on each gel. These markers were phosphorylase *b* (98 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and cytochrome *c* (12.3 kDa). Fluorography was conducted with the dimethyl sulfoxide–2,5-diphenyloxazole system described by Bonner and Laskey (3). All fluorograms were obtained with Eastman Kodak (Rochester, N.Y.) XAR5 X-ray film at exposure times of between 3 to 5 days at −70°C. Quantitative densitometric analyses of the fluorograms were made with a Hoefer GS 300 densitometer (Hoefer Scientific Instruments, San Francisco, Calif.).

Nitrite and protein assays. Nitrite was determined colorimetrically as described previously (7). Protein concentrations were determined by the biuret assay (6) after solubilization of cell protein in aqueous 3 N NaOH (30 min at 60°C) and sedimentation of insoluble material by centrifugation (14,000 × *g*, 5 min).

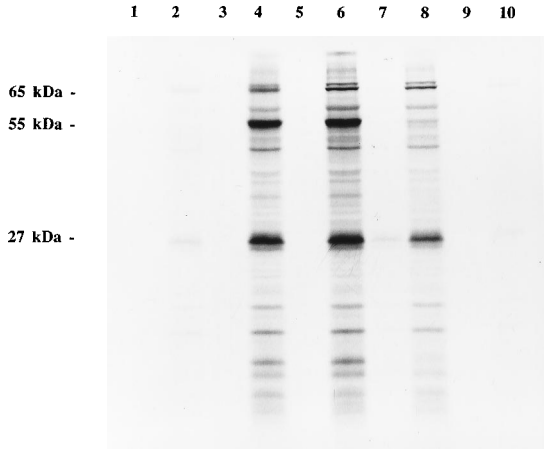

FIG. 1. ^{14}C radiolabeling of polypeptides from *N. europaea* in the presence of potential energy sources. Cells were incubated with $Na_2{}^{14}CO_3$, and electrophoresis and fluorography were conducted as described in Materials and Methods. The figure shows the fluorogram obtained for cells incubated with no additions (lane 1), 10 mM $NH_4{}^+$ (lane 2), 2 mM NH_2OH (lane 3), 10 mM $NH_4{}^+$ and 2 mM NH_2OH (lane 4), 2 mM N_2H_4 (lane 5), 2 mM N_2H_4 and 10 mM $NH_4{}^+$ (lane 6), 2 mM *n*-methylhydroxylamine (lane 7), 2 mM *n*-methylhydroxylamine and 10 mM $NH_4{}^+$ (lane 8), 2 mM *o*-methylhydroxylamine (lane 9), and 2 mM *o*-methylhydroxylamine and 10 mM $NH_4{}^+$ (lane 10). The positions and molecular masses of the major radiolabeled polypeptides are indicated on the left and were calculated by comparison with R_f values for molecular mass markers as described in Materials and Methods.

RESULTS

Our initial experiments aimed to determine which HAO substrates would make appropriate energy sources to support protein synthesis in the absence of ammonia oxidation. Acetylene-treated cells with $Na_2{}^{14}CO_3$ and C_2H_2 were incubated in the absence of exogenous $NH_4{}^+$, with 10 mM $NH_4{}^+$ [5 mM $(NH_4)_2SO_4$] alone or with 2 mM hydroxylamine hydrochloride, hydrazine hydrochloride, *n*-methylhydroxylamine hydrochloride, or *o*-methylhydroxylamine hydrochloride, all with and without 10 mM $NH_4{}^+$. Little or no incorporation of radiolabel into any polypeptide was observed for cells incubated without any additions, with $NH_4{}^+$ alone, or with each HAO substrate alone (2 mM) (Fig. 1). In contrast, high levels of incorporation of radiolabel into polypeptides were observed in most cases in

which cells were incubated with NH_4^+ and an energy source. For example, cells incubated with 10 mM NH_4^+ and either hydroxylamine, hydrazine, or *n*-methylhydroxylamine all exhibited very similar patterns of incorporation of radiolabel. In the case of hydroxylamine- and hydrazine-containing incubations, there were three polypeptides which showed high levels of radiolabel incorporation. The molecular masses of these polypeptides were 27, 55, and 65 kDa.

The 27-kDa polypeptide in Fig. 1 is the same active site-containing 27-kDa polypeptide which is radiolabeled after inactivation of AMO in the presence of $^{14}C_2H_2$ (10). This claim is supported by the fact that the 27-kDa polypeptide underwent an aggregation reaction when the protein samples were heated in the presence of SDS-PAGE sample buffer containing β-mercaptoethanol (data not shown). We have recently demonstrated that this phenomenon can be used as a diagnostic feature of AMOa in *N. europaea* and other autotrophic nitrifiers (11). The identities of the two other major polypeptides in Fig. 1 are not known. Differing levels of incorporation of radiolabel were observed with the different HAO substrates used to supply energy to support protein synthesis (Fig. 1). These differences may be due to a variety of causes, including different rates of substrate utilization and different coupling efficiencies of electron transport to ATP synthesis. In view of these complications, we chose to use hydroxylamine as the preferred energy source in subsequent experiments. Hydroxylamine is the natural energy source for *N. europaea*, and it is easy to establish the levels of hydroxylamine utilization by determining the nitrite levels in the incubation supernatant. The optimal conditions for labeling polypeptides using hydroxylamine as an energy source were subsequently determined. A time course of the labeling reaction demonstrated that incorporation of radiolabel into all polypeptides was complete within 30 min for cells incubated with 2 mM hydroxylamine and 10 mM NH_4^+. The optimal concentration of hydroxylamine for labeling was also determined to be 2 mM (data not shown).

The fluorogram shown in Fig. 2 demonstrates the effects of translation and transcription inhibitors on the ^{14}C radiolabeling reaction. In all incubations, there was complete utilization of all the exogenous energy source, hydroxylamine, as determined by the stoichiometric quantities of nitrite production. There were no ^{14}C-radiolabeled polypeptides detected in the samples of cells incubated with $Na_2{}^{14}CO_3$ and 2 mM hydroxylamine alone. In contrast, the three major radiolabeled polypeptides (with sizes of 27, 55, and 65 kDa) were observed for cells incubated with 10 mM NH_4^+ and 2 mM hydroxylamine. A low level of only one ^{14}C-radiolabeled polypeptide (size, 27 kDa) was observed for cells incubated with NH_4^+ and hydroxylamine in the presence of the transcriptional inhibitor rifampin (400 μg/ml). No ^{14}C radiolabeling of any polypeptide was observed under the same conditions when the translational inhibitor chloramphenicol (400 μg/ml) or the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (10 μM) was used rather than rifampin. Taken together, these results indicate that the radiolabeling reaction described in this study is energy dependent and involves de novo protein synthesis rather than posttranslational modification of preexisting polypeptides. The complete inhibition of radiolabeling of the 55- and 65-kDa polypeptides and the attenuated production of the 27-kDa polypeptide in the presence of rifampin also suggest that the synthesis of the radiolabeled polypeptides occurs in response to de novo gene transcription. The incomplete inhibition of de novo protein synthesis by high concentrations of rifampin is unlikely to reflect the presence of endogenous mRNA, since complete degradation of mRNA encoding AMO is known to occur within 3 h (25a). We have also observed the same incomplete inhibition of protein synthesis by rifampin in labeling reactions with cells stored in the dark at 4°C for 24 h (data not shown). Furthermore, our previous studies have also demonstrated incomplete inhibition of de novo protein synthesis in the presence of rifampin (10). This partial inhibition is compatible with the limited and selective ability of this organism to take up organic compounds (10, 20).

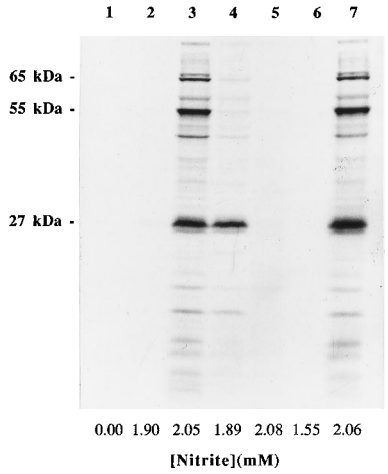

FIG. 2. The effect of inhibitors on the incorporation of ^{14}C into polypeptides. Cells were incubated with $Na_2{}^{14}CO_3$, and electrophoresis and fluorography were conducted as described in Materials and Methods. The figure shows the fluorogram obtained for cells incubated with no additions (lane 1), 2 mM NH_2OH (lane 2), and 10 mM NH_4^+ and 2 mM NH_2OH (lane 3). The remaining lanes are as described for lane 3, but with rifampin (400 μg/ml) (lane 4), chloramphenicol (400 μg/ml) (lane 5), carbonyl cyanide *m*-chlorophenylhydrazone (10 μM) added as a solution in dimethyl sulfoxide (10 μl) (lane 6), and dimethyl sulfoxide alone (10 μl) (lane 7). The positions and molecular masses of the major radiolabeled polypeptides are indicated on the left and were calculated by comparison with R_f values for molecular mass markers as described in Materials and Methods. The concentration of nitrite detected in each incubation is shown below the corresponding gel lane.

The effects of the NH_4^+ concentration on the ^{14}C radiolabeling reaction were investigated with cells incubated with $Na_2{}^{14}CO_3$, C_2H_2, one of two concentrations of hydroxylamine (0.5 and 2 mM), and a range of NH_4^+ concentrations up to 50 mM. The fluorogram (Fig. 3A) shows the ^{14}C radiolabeling pattern for cells incubated with the indicated range of NH_4^+ concentrations and 2.0 mM hydroxylamine. In each incubation, there was a complete utilization of all added hydroxylamine, as demonstrated by the constant concentration of nitrite detected in each incubation. Increases in NH_4^+ concentration led to increases in the level of ^{14}C incorporation into the three major polypeptides (sizes, 27, 55, and 65 kDa) (Fig. 3A). A densitometric analysis of the levels of ^{14}C incorporation into the 27-kDa polypeptide in the presence of 2.0 mM hydroxylamine was conducted, and these data are plotted versus NH_4^+ concentrations in Fig. 3B. This figure also shows the equivalent data obtained for cells incubated with 0.5 mM hydroxylamine. In both experiments, the densitometry shows that the level of ^{14}C incorporation was a saturable process. A computer fit of the data to a hyperbola provides an estimate for the K_s of 1.3 ± 0.2 and 2.4 ± 0.3 mM for NH_4^+ in incubations conducted with 0.5 and 2 mM hydroxylamine, respectively. Our best estimate for a true K_s value is approximately 2 mM NH_4^+, and this is based on results obtained for incubations conducted with optimal concentrations of hydroxylamine (2 mM).

The effects of the NH_4^+ concentration on the ^{14}C radiolabeling reaction were also investigated to determine whether

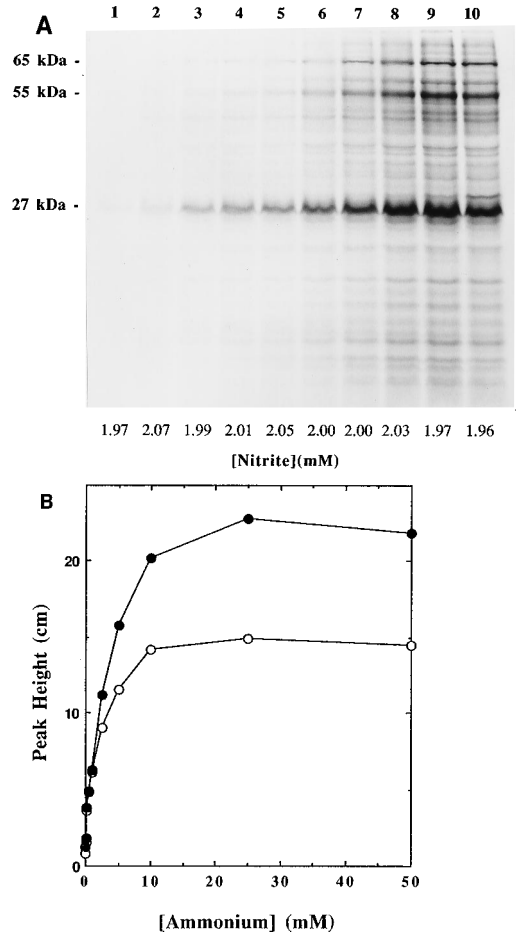

FIG. 3. The effect of NH_4^+ concentration on the incorporation of ^{14}C into the 27-kDa polypeptide. Cells were incubated with $Na_2{}^{14}CO_3$, and electrophoresis and fluorography were conducted as described in Materials and Methods. (A) The figure shows the fluorogram obtained for cells incubated with 2 mM NH_2OH and no NH_4^+ (lane 1) and with NH_4^+ at 0.1 mM (lane 2), 0.25 mM (lane 3), 0.5 mM (lane 4), 1.0 mM (lane 5), 2.5 mM (lane 6), 5.0 mM (lane 7), 10 mM (lane 8), 25 mM (lane 9), and 50 mM (lane 10). The positions and molecular masses of the major radiolabeled polypeptides are indicated on the left and were calculated by comparison with R_f values for molecular mass markers as described in Materials and Methods. The concentration of nitrite detected in each incubation is shown below the corresponding gel lane. (B) The figure shows the results of a densitometric analysis of the intensity (peak height) of the 27-kDa polypeptide from the fluorogram in Fig. 3A plotted versus the ammonium concentration (●). An experiment identical to the one described above for Fig. 3A was also conducted with the same cell preparation and 0.5 mM NH_2OH as the energy source. The results of that densitometric analysis are also included (○).

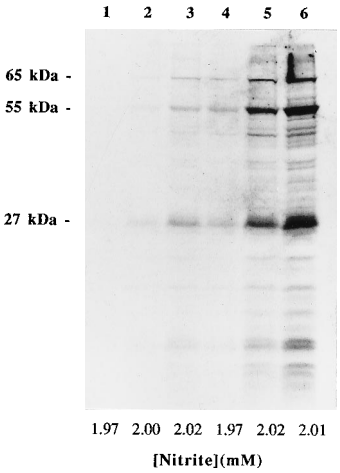

FIG. 4. The effect of pH and ammonia concentration on the ^{14}C radiolabeling reaction. Cells were incubated with $Na_2{}^{14}CO_3$, and electrophoresis and fluorography were conducted as described in Materials and Methods. The figure shows the fluorogram obtained for cells incubated with 2 mM NH_2OH and 1 mM NH_4^+ (pH 7.0) (lane 1), 1 mM NH_4^+ (pH 7.5) (lane 2), 1 mM NH_4^+ (pH 8.0) (lane 3), 10 mM NH_4^+ (pH 7.0) (lane 4), 10 mM NH_4^+ (pH 7.5) (lane 5), and 10 mM NH_4^+ (pH 8.0) (lane 6). The positions and molecular masses of the major radiolabeled polypeptides are indicated on the left and were calculated by comparison with R_f values for molecular mass markers as described in Materials and Methods. The concentration of nitrite detected in each incubation is shown below the corresponding gel lane.

the cells were responding to either the NH_3 or the NH_4^+ concentration. To examine this, a series of incubations with $Na_2{}^{14}CO_3$ and C_2H_2 were conducted with cells incubated with 2 mM hydroxylamine and either 1 or 10 mM NH_4^+. To vary the NH_3 concentration in solution, the pH of the incubation solution was adjusted to 7.0, 7.5, and 8.0. In all cases, there was complete oxidation of the hydroxylamine added to the incubation, as determined by the quantitative production of nitrite in each reaction. The level of ^{14}C incorporation increased for the 27-kDa polypeptide in step with increases in pH for cells incubated with either 1 or 10 mM NH_4^+ (Fig. 4). This experiment also demonstrated that the amounts of ^{14}C incorporation into the 27-kDa polypeptide were similar to those observed for cells incubated at pH 8.0 with 1 mM NH_4^+ and at pH 7.0 with 10 mM NH_4^+. Under these circumstances, the cells are theoretically exposed to equivalent concentrations of free NH_3 in solution. Taken together, the results presented in Fig. 3 and 4 suggest that the level of incorporation of radiolabel into the 27-kDa polypeptide is determined by the free NH_3 concentration rather than the ambient NH_4^+ concentration.

Other potential compounds which might be capable of influencing the synthesis and ^{14}C radiolabeling of the 27-kDa and other polypeptides were also investigated. We were unable to demonstrate the synthesis of the 27-kDa polypeptide or any other polypeptide when cells were incubated with $Na_2{}^{14}CO_3$, C_2H_2, 2 mM hydroxylamine, and other compounds such as alternative nitrogen sources, known AMO substrates, or known AMO inhibitors. These compounds included urea (10 mM), methylamine (10 mM), glutamate (2 mM), glutamine (2 mM), nitrogen trifluoride (25% [vol/vol], gas phase), methane (50% [vol/vol], gas phase), and methyl fluoride (10% [vol/vol], gas phase). We also considered whether ^{14}C radiolabeling of specific polypeptides could be achieved under anaerobic conditions. The best-characterized anaerobic processes in *N. europaea* are those involving the reduction of nitrite to nitrous oxide (1, 23, 25), and both hydrazine (25) and pyruvate (1) are known to support nitrite-dependent nitrous oxide production. The results shown in Fig. 5 demonstrate that the 27-kDa polypeptide was synthesized in the presence of NH_4^+ when either hydrazine or pyruvate was used to support the anaerobic reduction of nitrite.

DISCUSSION

The major aim of this study was to investigate the effects of ammonia on de novo protein synthesis in cells incapable of using ammonia as an energy source. There are well-defined approaches to investigating these types of physiological questions in heterotrophic bacteria. However, in the case of autotrophic ammonia oxidizers, the methods required to answer these physiological questions are more complex. The most

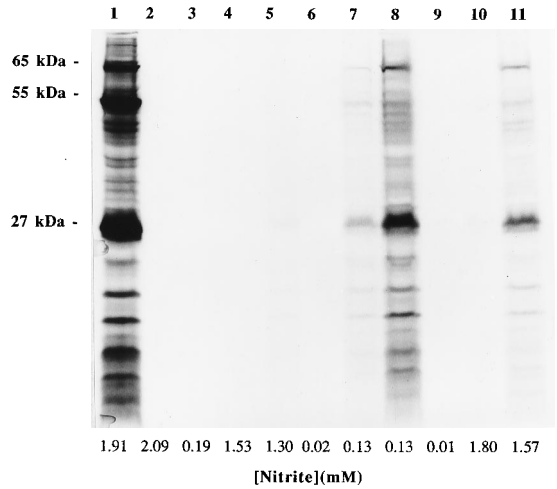

FIG. 5. Synthesis of polypeptides under anaerobic conditions. Cells were incubated with $Na_2{}^{14}CO_3$ under anaerobic conditions for 2 h. All other aspects of the incubations, electrophoresis, and fluorography were conducted as described in Materials and Methods. The figure shows the fluorogram obtained for cells incubated with 2 mM NH_2OH and 10 mM NH_4^+ under aerobic conditions (lane 1); 2 mM NO_2^- (lane 2); 2 mM NH_2OH (lane 3); 2 mM NO_2^- and 2 mM NH_2OH (lane 4); 2 mM NO_2^-, 2 mM NH_2OH, and 10 mM NH_4^+ (lane 5); 2 mM N_2H_4 (lane 6); 2 mM NO_2^- and 2 mM N_2H_4 (lane 7); 2 mM NO_2^-, 2 mM N_2H_4, and 10 mM NH_4^+ (lane 8); 2 mM pyruvate (lane 9); 2 mM pyruvate and 2 mM NO_2^- (lane 10); and 2 mM NO_2^-, 2 mM pyruvate, and 10 mM NH_4^+ (lane 11). The positions and molecular masses of the major radiolabeled polypeptides are indicated on the left and were calculated by comparison with R_f values for molecular mass markers as described in Materials and Methods. The concentration of nitrite detected in each incubation is shown below the corresponding gel lane.

significant problems are that these organisms are both fastidious lithotrophs and autotrophs. Furthermore, they appear to express AMO constitutively, although large fluctuations in AMO activity have been described (10). For these reasons, it is not possible, for example, to follow protein synthesis when heterotrophically grown cells are transferred to autotrophic conditions in the presence of such protein-labeling compounds as [^{35}S]methionine. In view of these constraints, alternative experimental approaches have been required.

The most critical aspect of the approach used in this study involves the highly specific effects of acetylene as a mechanism-based inactivator of AMO. The continuous presence of acetylene in our experiments served two purposes. First, acetylene fully inactivated any preexisting AMO. Second, the continual presence of acetylene assures that any newly synthesized AMO becomes irreversibly inactivated before it can oxidize appreciable amounts of ammonium. In combination, these two effects prevent the cells from utilizing ammonium as an energy source while the cells are exposed to ammonium ions. Although the inability of cells to utilize exogenous ammonium as an energy source is predicted by the presence of acetylene, this effect is also confirmed by the stoichiometric production of nitrite from the known concentrations of hydroxylamine added to most incubations. If cells had retained the ability to oxidize ammonium, nitrite concentrations in ammonium- and hydroxylamine-containing reactions would have exceeded the quantity of nitrite expected from the quantity of hydroxylamine added. This effect was not observed in our experiments.

The use of limiting concentrations of hydroxylamine in our experiments also has the important role of equalizing the quantity of energy available to cells during the ^{14}C radiolabeling reactions. This feature has allowed us to interpret our results specifically in terms of the effects of exogenous stimuli and inhibitors without the added complication of assessing the significance of various levels of substrate oxidation on the total energy budget of the cells. An unforeseen feature of the ^{14}C radiolabeling technique we have described is that this provides a very sensitive method to detect energy-generating reactions within *N. europaea*. The ability of cells to oxidize other substrates for HAO in the presence of NH_4^+ and to synthesize and incorporate ^{14}C into the same polypeptides synthesized in the presence of NH_4^+ and hydroxylamine (Fig. 1) suggests that the oxidation of that compound is coupled to ATP synthesis. Our results demonstrate that this is certainly true of the aerobic oxidation of the known HAO substrates hydroxylamine, hydrazine, and *n*-methylhydroxylamine (9) (Fig. 1). Perhaps more interesting, our results with hydrazine and pyruvate under anaerobic conditions (Fig. 5) also demonstrate that the reduction of nitrite is an energy-yielding reaction for *N. europaea*. However, the low level of incorporation of radiolabel that we observed with these electron donors suggests that neither of these processes is either particularly energetically efficient or rapid compared with energy generation under aerobic conditions. ^{14}C uptake from $^{14}CO_2$ has been reported previously for cells incubated with pyruvate and nitrite (1) but not for cells incubated with hydrazine and nitrite. In view of the difficulties involved in culturing ammonia oxidizers under anything less than optimal aerobic conditions, the ability to incorporate $^{14}CO_2$ into defined polypeptides could provide a very convenient method to investigate other potential electron acceptor and donor couples for this organism.

One of the most significant observations is that acetylene-treated cells synthesize the 27-kDa component of AMO in response to the presence of NH_4^+. Potentially, this effect may reflect either an induction of de novo protein synthesis in response to gene transcription or an effect of ammonia as an N source for protein synthesis. Although our results do not allow us to unequivocally assess the contribution of each of these potential roles to this system, several findings suggest that ammonia contributes significantly to the regulation of de novo synthesis of the 27-kDa component of AMO and other polypeptides. Our evidence supporting this conclusion includes the observation that the 27-kDa component of AMO is only synthesized in the presence of ammonia (Fig. 1) and that this response is inhibited by protein synthesis and transcriptional inhibitors (Fig. 2). We have also observed that this is a saturable response with respect to ammonium concentration and that the cells respond to ammonia (NH_3) rather than ammonium (NH_4^+). An analysis of the levels of ^{14}C incorporation into the 27-kDa polypeptide of AMO indicates that the synthesis of this polypeptide is a saturable response with a half-maximal response (K_s) at pH 7.8 of approximately 2 mM NH_4^+ (Fig. 3). Our results also suggest that the cells respond to NH_3 rather than NH_4^+ (Fig. 4). Assuming a pK_a for NH_4^+ of 9.3 at 30°C, this value can be recalculated in terms of free NH_3 at approximately 60 μM NH_3. It is interesting to note that this K_s value for NH_3 is close to the reported K_m for NH_3 for AMO of 30 μM and that it is NH_3, rather than NH_4^+, which is regarded as the true substrate for this enzyme (26). The similarity between the K_s for this response and the K_m for AMO for ammonia suggests that the cells are capable of regulating the expression of this enzyme over a physiologically relevant concentration range. The induction of other gas-utilizing enzymes is also known to be dependent on the concentrations of their substrates. For example, the synthesis of a component of an uptake hydrogenase is dependent on the H_2 concentration when heterotrophically grown cells of *Alcaligenes latus* are transferred to autotrophic conditions (4). This saturable process is regulated over 5 orders of magnitude in H_2 concentra-

tion (0.001 to 30%, vol/vol). Cells of *Rhodospirillum rubrum* respond to CO by synthesizing a nickel-free form of carbon monoxide dehydrogenase in nickel-free media (2). This example is closely analogous to our present experiments, because nickel-free carbon monoxide dehydrogenase is catalytically inactive and yet cells synthesize large quantities of this enzyme without the ability to benefit from the activity of this enzyme.

An important observation from our experiments is that we have been unable to demonstrate the synthesis of any polypeptides in the presence of compounds other than NH_3. These compounds included potential alternative nitrogen sources and known alternative substrates and inhibitors of AMO. This result may simply reflect the fact that the cells have an absolute need for ammonia as an N source for protein synthesis. While a requirement for an N source is expected to influence our observations, evidence suggests an additional and more significant role for ammonia as an inducer of AMO synthesis. For example, we have observed that the same polypeptides are synthesized under both aerobic and anaerobic conditions. In the case of cells incubated with pyruvate and nitrite (Fig. 5), it has previously been shown that cells of *N. europaea* can produce significant quantities of protein in the absence of exogenous ammonia (1). This observation suggests that cells of *N. europaea* do not have an absolute requirement for exogenous ammonia as an N source for protein synthesis. Furthermore, the K_m for NH_4^+ for glutamate dehydrogenase, the only nitrogen-assimilating enzyme which has been studied in detail in *N. europaea*, is 16 mM at pH 7.5 (8). This value is far higher than the K_s for NH_4^+ we have determined, and this suggests that the effects of NH_4^+ concentration on gene transcription and N assimilation are likely to be independent.

A final important observation is that although the 27-kDa polypeptide is one of the major polypeptides synthesized under our experimental conditions, it is also important to note that two additional major (55- and 65-kDa) and several minor polypeptides are also usually synthesized. This suggests that the proposed inductive effect of ammonia is not entirely specific for AMO and may involve a more global response. The identities and roles of the two additional major radiolabeled (55- and 65-kDa) polypeptides are currently unknown. Because these polypeptides are not consistently synthesized in stoichiometric quantities with respect to the 27-kDa polypeptide, it seems they are unlikely to be additional structural components of AMO. However, no polypeptide corresponding to AMOb (ca. 40 kDa) was synthesized in our experiments. Because AMOa and AMOb are present in about equal concentrations in purified forms of acetylene-inactivated AMO (22), the unequal labeling in our experiments may indicate that the synthesis of AMO components may not be synchronous.

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