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Regulation of Nitrogenase Activity by Ammonium Chloride in *Azospirillum* spp.

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Ammonium chloride (≥0.05 mM) effectively and reversibly inhibited the nitrogenase activity of *Azospirillum brasilense*, *Azospirillum lipoferum* and *Azospirillum amazonense*. The glutamine synthetase inhibitor L-methionine-DL- sulfoximine abolished this "switch-off" in *A. lipoferum* and *A. brasilense*, but not in *A. amazonense*. Azaserine, an inhibitor of glutamate synthase, inhibited nitrogenase activity itself. This provides further evidence for glutamine as a metabolite of regulatory importance in the NH_4^+ switch-off phenomenon. In *A. brasilense* and *A. lipoferum*, a transition period before the complete inhibition of nitrogenase activity after the addition of 1 mM ammonium chloride was observed. The in vitro nitrogenase activity also was decreased after treatment with ammonium. During sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a second dinitrogenase reductase (Fe protein) subunit appeared, which migrated in coincidence with the modified subunit of the inactive Fe protein of the nitrogenase of *Rhodospirillum rubrum*. After the addition of ammonium ^{32}P was incorporated into this subunit of the Fe protein of *A. brasilense*. In *A. amazonense*, the inhibition of nitrogenase activity by ammonium was only partial, and no transition period could be observed. The in vitro nitrogenase activity of ammonium-treated cells was not decreased, and no evidence for a modified Fe protein subunit was found. Nitrogenase extracts of *A. amazonense* were active and had an Fe protein that migrated as a close double band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The nitrogenase reaction is an energy-demanding process subject to sophisticated regulatory controls. Besides the regulation of nitrogenase synthesis at the level of *nif* gene transcription by the combined nitrogen, oxygen, and temperature (32), a posttranslational regulation of nitrogenase activity has been described.

The rapid and reversible inhibition of nitrogenase activity by small extracellular concentrations of ammonium has been termed "NH_4^+ switch-off" and has been studied in a variety of organisms (4, 14, 16, 26, 31, 33, 34, 37). A covalent modification of the dinitrogenase reductase (Fe protein) was found to inactivate nitrogenase in response to the addition of ammonium (15, 16). The modifying group of the inactive Fe protein of *Rhodospirillum rubrum* is ADP-ribose (31), and an activating enzyme from *R. rubrum* for the removal of the group has been purified to homogeneity (33). The sensitivity to NH_4^+ switch-off and the extent of the Fe protein inactivation is dependent on cultural conditions (10) and strain, e.g., in *Azotobacter vinelandii* (9, 19, 21). A covalent modification of the Fe protein from *Chromatium vinosum* after the addition of ammonium was described recently (10).

The short-term inhibition of nitrogenase activity by ammonium has been observed, but not well characterized, for most other microorganisms. The use of mutant organisms and analogs of amino acids as inhibitors of glutamine synthetase (GS) (e.g., L-methione-DL-sulfoximine [MSX]) and glutamate synthase (e.g., azaserine [2, 7]) suggest that glutamine is a mediator of ammonium inhibition of nitrogenase. *Azospirillum lipoferum* and *Azospirillum brasilense* (35) have attracted research efforts because they are able to fix nitrogen and to colonize the root surface or invade the roots of important forage and grain crops (6, 27). *Azospirillum* spp. fix nitrogen only under microaerobic conditions (29, 36). *Azospirillum* spp. use organic acids readily (e.g., malate, lactate, or pyruvate), and some utilize sugars (35). *Azospirillum amazonense* has a considerably lower pH optimum (pH 6) than that of other species, and it can grown and fix nitrogen with sucrose as its carbon source (24).

The general properties of the nitrogen assimilation system (28, 36) and the nature of mutants in the genes for GS (7), glutamate synthase (2) and nitrogenase (14, 30) of the azospirilla have been reported. Ludden et al. (23) studied the nitrogenase system of *Spirillum lipoferum* (*A. brasilense* Sp7). Its nitrogenase system is a three-component system (dinitrogenase, dinitrogenase reductase, and activating enzyme), and the dinitrogenase reductase (Fe protein) normally is isolated in an inactive form. It can be activated by its own activating enzyme in a manganese-dependent reaction, or by the activating enzyme of *R. rubrum*. Nair et al. (25) demonstrated two Fe protein subunits of *A. brasilense* on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the Western blotting technique; the subunits resembled those of the inactive Fe protein of *R. rubrum* (16).

In this communication, we report the rapid inhibition by ammonium of nitrogenase in *A. brasilense* Sp7, *A. lipoferum* SpRG20a and SpBr17, and *A. amazonense* Y1. The nitrogenase of *A. brasilense* and *A. lipoferum* is regulated by the covalent modification of the Fe protein, whereas in *A. amazonense* a different, noncovalent inhibitory mechanism caused only a partial inhibition of nitrogenase activity by ammonium.

MATERIALS AND METHODS

Organisms and conditions of culture. *A. brasilense* Sp7 (ATCC 29145), *A. lipoferum* SpRG20a (ATCC 29708), and SpBr17 (ATCC 29709) were obtained from the American Type Culture Collection. *A. amazonense* Y1 (type strain) was kindly provided by N. R. Krieg.

A. brasilense and *A. lipoferum* strains were grown in

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minimal salt medium (including microelements) as described by Albrecht and Okon (1). *A. amazonense* Y1 was grown in minimal medium (pH 6.0) according to Magalhaes et al. (24) with morpholineethanesulfonic acid (MES; 50 mM) added to stabilize the pH. To obtain exponentially growing nitrogen-fixing cultures (2 liter batch), N-free minimal medium was inoculated with a 200-ml culture that had grown for 24 h on 20 mM ammonium chloride. After the ammonium was diluted from the inoculum by transfer to the 2-liter culture and subsequently depleted, the growth slowed (nitrogen fixation [29]). For comparison, 0.75% malate was used as the carbon source for all three strains, and HCl was added through a pH-stat. The dissolved-oxygen concentration was measured continuously and was controlled at 0.003 to 0.006 atm (1 atm = 101.29 kPa) by an oxystat.

For ^{32}P labeling, the potassium phosphate buffer was replaced by morpholinepropanesulfonic acid (MOPS) buffer (100 mM, pH 6.8) and potassium chloride (50 mM). The initial phosphate concentration was 0.4 mM, and 1 mCi of carrier-free $^{32}PO_4{}^{3-}$ was added after inoculation. The generation time was 5 h at 0.003 atm of dissolved oxygen for nitrogen-fixing cultures of *A. brasilense* Sp7. The whole-cell nitrogenase activity was 500 nmol of ethylene h^{-1} ml of culture^{-1} (A_{580} = 1).

Whole-cell nitrogenase activity. Nitrogenase activity was measured in serum bottles or a stirred chamber. Serum bottles were evacuated and refilled repeatedly with argon, and then the culture samples (2 ml, leaving 21 ml of gas space) were injected. The air supplement to support optimal nitrogenase activity was determined; 0.006 to 0.012 atm of oxygen was supplied in the gas phase, depending on the culture density and strain. The bottles were shaken at 160 strokes min^{-1}. Alternatively, a vigorously stirred 30-ml chamber (13) equipped with a Clark-type oxygen electrode was used. After flushing with N_2 to achieve anaerobiosis, culture samples (4 ml) were injected, and the dissolved-oxygen concentration was adjusted to the desired level by injecting air; 0.001 to 0.002 atm (0.1 to 0.2 kPa) of dissolved oxygen was optimal. The dissolved-oxygen concentration was maintained by repeated injections of 50 to 100 μl of air. The reaction was run at 30°C and was started by adding acetylene (0.1 atm). Gas samples (0.2 or 0.5 ml) were withdrawn periodically and analyzed gas chromatographically with a flame ionization detector (3).

Oxygen uptake rates. Dissolved-oxygen concentrations were followed continuously during nitrogenase assays in the rapidly mixed chamber, and oxygen consumption rates per milligram of cell protein were calculated. These values were the same as those obtained in other experiments with an oxygen electrode and were compatible with published respiration rates of N_2-fixing azospirilla (28, 36).

Preparation of crude extracts. Samples of cultures (450 ml) were siphoned into centrifuge bottles under a continuous stream of N_2, and the sealed bottles were centrifuged at 7,000 × *g* for 10 min at 4°C. The cell pellet was suspended under N_2 in 5 ml of 500 mM Tris acetate buffer (pH 8.5, containing 8 mM dithionite and 1 mM dithiothreitol), and the anaerobic cell suspension was frozen in liquid nitrogen. After incubation with lysozyme (1 mg/ml) at 4°C for 30 min, the cell suspension was sonicated with an Ultrasonic Cell Disruptor model 350 (Heat Systems-Ultrasonics, Inc.; output 4, 50% duty cycle) for 2 min at 4°C in an anaerobic chamber. After ultracentrifugation for 2 h at 125,000 × *g* at 4°C, the supernatant (extract) was stored in liquid nitrogen.

Alternatively, we quickly filtered (30 s) 10-ml samples of culture through glass microfiber filters (Whatman GF/A for *A. lipoferum* and GF/C for *A. brasilense*) or cellulose triacetate membrane filters (Gelman GA-3; pore size, 1.2 μm, for *A. amazonense*). The filters then were frozen quickly in liquid nitrogen, and the collected cells were ground quickly with carborundum in anaerobic buffer (16).

In vitro nitrogenase assay. The dithionite-dependent aceytlene reduction assay was performed with a 1-ml reaction mixture containing 100 μg of creatine kinase, 40 μmol of creatine phosphate, 15 μmol of magnesium acetate, 0.5 μmol of $MnCl_2$, 30 μmol of MOPS buffer (pH 7.0), 5 μmol of ATP, and 5 μmol of sodium dithionite. Acetylene to give 0.1 atm was injected into anaerobic vials, and the reaction was started by adding the nitrogenase extract (0.5 to 1.5 mg of protein). After 30 min of shaking at 160 complete strokes min^{-1} at 30°C, 0.3 ml of trichloroacetic acid (25%, wt/vol) was added to terminate the reaction. The gas phase was analyzed by gas chromatography, and protein concentrations were determined (8).

GS assay. The synthetic reaction of glutamine was determined by the method of Kleinschmidt and Kleiner (18), and activity measured in the presence of Mg^{2+} reflected the active GS enzyme.

Immunoprecipitation. The *Azospirillum* Fe protein was immunoprecipitated from "quick extracts" with antiserum against the *R. rubrum* Fe protein (kindly provided by P. W. Ludden). *Staphylococcus aureus* cells were used as an immunoadsorbent by the method of Cullen and Schwartz (5) with modifications. Protein-A containing *S. aureus* cells in MSP buffer (MOPS buffer [20 mM, pH 7.5] with 0.9% NaCl and 0.5% Nonidet P-40) were incubated for 60 min on ice with *Azospirillum* crude extract (3 mg of protein) to saturate unspecific binding sites. After centrifugation, the *S. aureus* cells were resuspended in MSP buffer. The ^{32}P-labeled quick extracts (0.5 ml) or the control samples were incubated for 30 min with 30 μl of Rr2 antiserum on ice and diluted with 0.5 ml of MSP buffer. Then 0.1 ml of preincubated *S. aureus* cells was added. After 60 min on ice, the cells were washed three times in ice-cold MSP buffer. To lower the labeling background, the vials were changed after the first centrifugation step. Finally, the pellet was suspended in Laemmli SDS sample buffer (19) and heated in a boiling water bath for 2 min. The tubes were centrifuged for 2 min, and the supernatant was used for SDS-PAGE.

SDS-PAGE. SDS-PAGE (22) was performed as modified by Kanemoto and Ludden (16) with acrylamide:N,*N'*-methylenebis(acrylamide), 30:0.174 (low cross-linker content), to obtain a better resolution of the Fe protein subunits. Proteins were mixed with 70 μl of SDS sample buffer (22), treated for 1 min in a boiling water bath, and then were loaded onto the stacking gel. The samples were run through the stacking gel at 150 V and the separating gel (0.3 by 13.5 by 9 cm) at 220 V. After protein staining with Coomassie brilliant blue (0.05% [wt/vol] Coomassie brilliant blue, 25% [vol/vol] ethanol, 5% [vol/vol] acetic acid), the gels were destained and dried. Autoradiographs of the dried gels were made at −80°C with intensifying screens and Kodak X-Omat AR film.

Enzyme-linked immunoblotting. Crude extracts (4 μg of protein) or purifed Fe protein of *R. rubrum* (0.025 μg of protein) were electrophoresed as described above. The protein was electroblotted from the polyacrylamide gel onto a nitrocellulose filter for 3 h in Tris glycine buffer (pH 8.3; 20% methanol, 25 mM Tris, 192 mM glycine) with 250 mA of constant current. After equilibration for 1 h with the blotting buffer (20 mM Tris, 500 mM sodium chloride, pH 7.6, with 5 g of skim milk per 100 ml), the filters were agitated gently

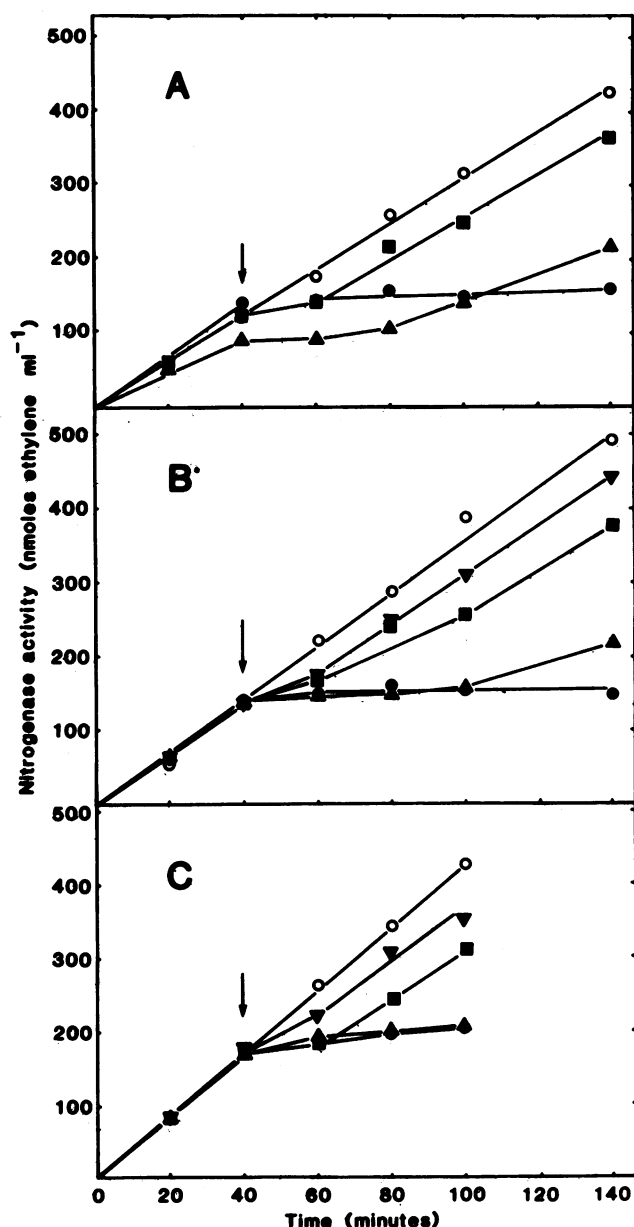

FIG. 1. NH_4^+ switch-off of the nitrogenase activity in *A. brasilense* Sp7 (A), *A. lipoferum* SpBr17 (B), and *A. amazonense* Y1 (C). Exponentially growing, nitrogen-fixing cultures (A_{580}, 1.3 to 1.5) were tested for nitrogenase activity with the acetylene reduction assay in rapidly shaking bottles (see the text). At 40 min (arrow) after the addition of acetylene, ammonium chloride was added at final concentration of 1 (●), 0.25 (▲), 0.10 (■), and 0.05 (▼) mM. As a control (○), the same volume of water was added.

overnight in blotting buffer with the antiserum or the preimmune serum at room temperature. After washing the filters for 10 min in Tris sodium chloride buffer (pH 7.6) containing 0.05% Tween 20 and then twice in Tris buffer without detergent, the incubation was continued for 1 h with goat anti-rabbit immunoglobulin G horseradish peroxidase conjugate. The filters were washed again, as described above, and were finally stained in 0.015% H_2O_2 in Tris sodium chloride buffer (pH 7.6) with 0.5 mg of horseradish peroxidase development reagent per ml for 15 min. The Fe protein subunits on wet nitrocellulose filters were scanned with an integrating Zeineh Soft Laser densitometer. The stained Fe protein gave a linear response for extract concentrations from 0.1 to 4 μg of total protein (data not shown).

Chemicals. All chemicals and gases used were of high purity or analytical grade from Bio-Rad Laboratories, Sigma Chemical Co., United States Biochemical Corp., Boehringer Mannheim Biochemicals, Aldrich Chemical Co., or New England Nuclear Corp.

RESULTS

The concentration-dependent NH_4^+ switch-off of nitrogen fixation in *A. brasilense* Sp7, *A. lipoferum* SpBr17 and SpRG20a, and *A. amazonense* Y1 is illustrated in Fig. 1A through C. The addition of 50 or 100 μM ammonium chloride caused a transient inhibition of nitrogenase activity, and higher ammonium concentrations extended the inhibition period. With 0.25 and 1 mM NH_4Cl, a rapid and total inhibition of nitrogenase activity occurred in *A. brasilense* (Fig. 1A) and *A. lipoferum* (Fig. 1B), whereas in *A. amazonense*, the inhibition was not complete (Fig. 1C).

The addition of MSX, an inhibitor of GS, abolished the nitrogenase inhibition by ammonium chloride in *A. lipoferum* SpBr17 (Fig. 2A) and *A. brasilense* Sp7 (data not shown). When MSX was supplied after the addition of ammonium, the nitrogenase switch-off was reversed. Lower nitrogenase activity in the presence of MSX and NH_4^+, as compared with that of the control without any addition (Fig. 2A), also was found in the presence of MSX alone (data not shown). Azaserine (1 mM), an inhibitor of glutamate synthase, strongly inhibited nitrogenase activity, perhaps by increasing the cellular glutamine level; it did not relieve the ammonium inhibition of nitrogenase activity. Glutamine (1 mM), like ammonium, inhibited nitrogenase activities in all three strains. The addition of MSX had no effect on the nitrogenase inhibition by glutamine (data not shown).

In *A. amazonense* Y1, however, MSX could not abolish the NH_4^+ switch-off (Fig. 2B). In contrast to *A. brasilense*, the GS of *A. amazonense* was not inhibited in cells incubated with MSX (Table 1). When the inhibitory effect of MSX on GS was tested in vitro, the GSs of *A. brasilense* and *A. amazonense* were equally sensitive (Table 1). The ammo-

TABLE 1. Inhibition of glutamine synthetase activity by MSX in *A. brasilense* Sp7 and *A. amazonense* Y1

Treatment with MSX (mM)	GS activity (10^{-3} u/mg of protein):	
	A. brasilense Sp7	*A. amazonense* Y1
In vitro[a]		
0.0	86 (100[b])	130 (100)
0.05	69	117
0.20	39	82
1.0	28 (33)	45 (35)
In vivo[c]		
0.0	75 (100)	118 (100)
0.01	59	107
0.10	31 (41)	109 (92)

[a] The Mg-dependent synthetic assay for GS (18) was performed with extracts prepared from nitrogen-fixing cultures with different concentrations of MSX in the assay.

[b] Percentages are given in parentheses.

[c] Cell suspensions (150 ml) from nitrogen-fixing cultures were incubated for 5 min at 30°C with different concentrations of MSX, rapidly chilled in ice, and washed twice in cold 100 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (pH 7.5). Finally, the cells were suspended in 0.7 ml of buffer; crude extracts were prepared by sonication; and the GS activity was tested.

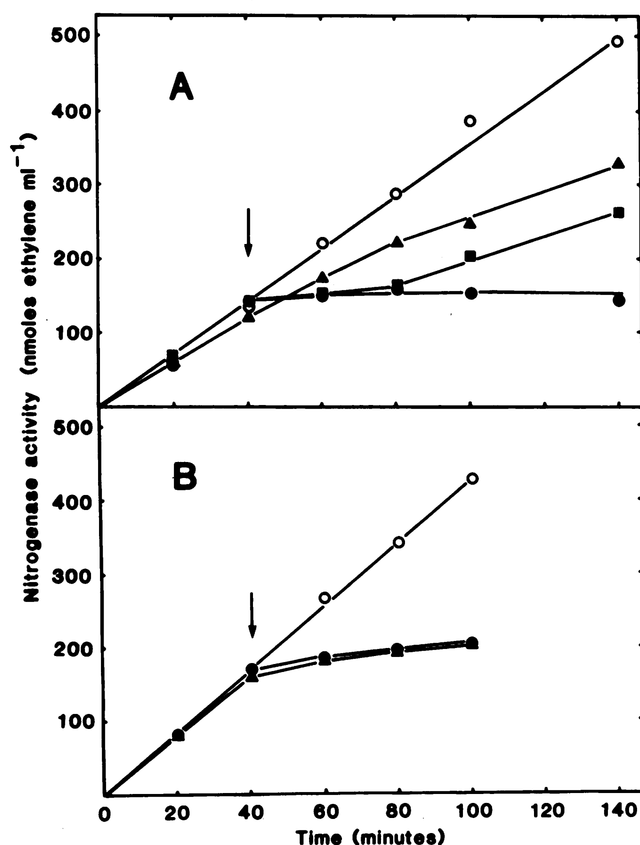

FIG. 2. Effects of MSX on the NH_4^+ switch-off of nitrogenase activity in *A. lipoferum* SpBr17 (A), and *A. amazonense* Y1 (B). The experiments were performed as described in the legend to Fig. 1. At 20 min before (▲) or after (■) the addition of 1 mM ammonium chloride (arrow), 0.1 mM MSX was injected. As controls, ammonium chloride (●) or the same volume of water (○) was given at 40 min.

nium uptake (measured as [^{14}C]methylammonium uptake) of the *A. amazonense* cells was less inhibited than was that of *A. brasilense* by MSX (data not shown). *A. amazonense* grew readily in the presence of MSX (0.1 mg/ml), whereas the growth of *A. brasilense* and *A. lipoferum* was totally inhibited by MSX (11). Perhaps *A. amazonense* Y1 takes up MSX poorly.

As an alternative to the experiments in rapidly shaken bottles (Fig. 1 and 2), the NH_4^+ switch-off experiments also were performed in an incubation chamber that allowed the continuous recording and regulation of the dissolved-O_2 concentration and the simultaneous measurement of the acetylene reduction activity (13). A rapidly spinning magnetic stirring bar quickly equilibrated O_2 between the gas and liquid phases, so that a lower concentration of oxygen (0.1 to 0.2 kPa of O_2) was needed to support optimal nitrogenase activity than that which was needed in the shaken bottles (0.8 to 1.2 kPa of O_2).

The time course of NH_4^+ switch-off in *A. brasilense* Sp7 was measured at different constant dissolved-oxygen concentrations (Fig. 3). At low oxygen concentrations (0.1 and 0.4 kPa of O_2), the nitrogenase activity decreased until the inhibition was almost complete in 10 to 15 min. At higher oxygen concentrations (0.6 to 1.5 kPa of O_2) that drastically inhibited nitrogenase activity, the inhibition by ammonium

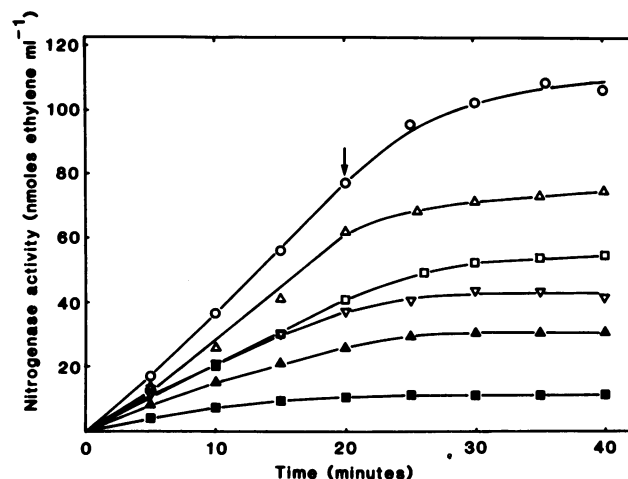

FIG. 3. Inhibition of nitrogenase activity of *A. brasilense* Sp7 at different dissolved-oxygen concentrations. Samples (4 ml) from an exponentially growing nitrogen-fixing culture (with 0.3 kPa of O_2 in equilibrium with solution) were transferred to an oxygen chamber, and the acetylene reduction activity was measured at 0.1 (○), 0.2 (△), 0.4 (□), 0.6 (▽), 1.0 (▲), and 1.5 (■) kPa of oxygen in equilibrium with solution (see the text). At 20 min (arrow) 1 mM ammonium chloride was injected.

was complete within 5 to 10 min. Similar results were obtained with *A. lipoferum* (data not shown). The time course of NH_4^+ switch-off in *A. amazonense* Y1 was quite different (Fig. 4). Immediately after the addition of ammonium chloride, nitrogenase activity slowed without the tran-

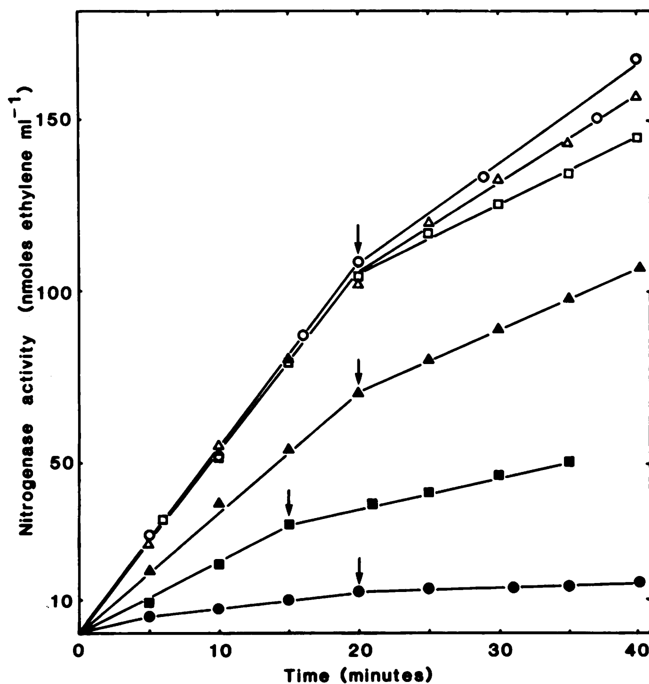

FIG. 4. Inhibition of nitrogenase activity in *A. amazonense* Y1 at different dissolved-oxygen concentrations. The acetylene reduction activity was measured at 0.04 (○), 0.2 (△), 0.4 (□), 1.0 (▲), 2.0 (■), and 4.0 (●) kPa of O_2 in equilibrium with the solution, as described in the legend to Fig. 3.

sition period observed with *A. brasilense* Sp7 and *A. lipoferum* SpBr17. At O_2 concentrations of 0.04 to 0.1 kPa, 55 to 60% of the activity remained after the addition of ammonium, whereas at 1.0 to 4.0 kPa of O_2, only about 30% of the activity remained. The pattern of inhibition of nitrogenase activity by ammonium was similar for *A. amazonense* Y1 grown and tested with sucrose, rather than malate, as the carbon source (data not shown).

The nitrogenase activity in cell extracts and the status of the Fe protein of nitrogenase were examined before and after the addition of ammonium. At the same time, whole-cell nitrogenase activities and oxygen uptake rates were tested in the controlled oxygen chamber at 0.2 kPa of dissolved oxygen (Table 2).

In *A. brasilense* and *A. lipoferum*, whole-cell nitrogenase activites decreased to about 1% at 15 min after the addition of 1 mM ammonium chloride. The in vitro nitrogenase activity also decreased in extracts prepared from culture samples taken after the addition of ammonium (Table 2). However, in *A. amazonense*, the addition of as much as 10 mM ammonium chloride reduced the whole-cell nitrogenase activity only partially, and the in vitro nitrogenase activity was not inhibited by the addition of ammonium to the culture. In response to the addition of 1 mM ammonium chloride, the rates of oxygen uptake in *A. brasilense* and *A. lipoferum* were increased, whereas in *A. amazonense*, the rate was unaltered (Table 2).

The status of the Fe protein of nitrogenase in cell extracts prepared from cultures before and after the addition of ammonium was examined with the enzyme-linked immunoblotting technique and with antiserum against the *R. rubrum* Fe protein (kindly provided by P. W. Ludden). *A. brasilense* and *A. lipoferum* showed two subunits of the Fe protein on SDS-PAGE; these migrated like the *R. rubrum* Fe protein

TABLE 2. Comparison of NH_4^+ switch-off effects in *Azospirillum* species

Strain and addition of NH_4^+ (1 mM)	Whole-cell nitrogenase activity (nmol of ethylene min^{-1} mg of protein^{-1})	Oxygen uptake of whole cells (nmol of O_2 min^{-1} mg of protein^{-1})	In vitro nitrogenase activity (nmol of ethylene min^{-1} mg of protein^{-1})	Ratio[a] of Fe protein subunits
A. brasilense Sp7				
Before	22	114	1.0	0.98
After	0.2 (0.9[b])	167	0.09 (9)	1.92
A. lipoferum SpRG20a				
Before	45	126	1.8	0.74
After	0.5 (1.0)	173	0.9 (50)	1.18
A. amazonense Y1				
Before	38	143	18	0.74[c]
After	13	138	22	0.65[c]
After[d]	10 (27)	138	NT[e]	NT

[a] The upper/lower subunit ratio was determined with the integrated peak areas of densitometer scans of Fe protein subunits in immunoblots of SDS-PAGE with extracts (see the text).
[b] Percentages are in parentheses.
[c] These subunits migrated distinctly differently than those of *A. brasilense*, *A. lipoferum*, or *R. rubrum*.
[d] 10 mM NH_4^+.
[e] NT, Not tested.

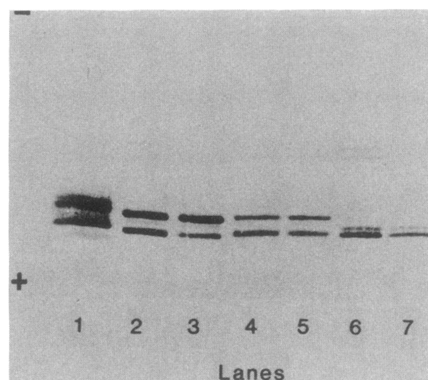

FIG. 5. Immunoblots with antiserum against Fe protein of *R. rubrum* and *Azospirillum* extracts separated by SDS-PAGE. Extracts of N_2-fixing *A. brasilense* Sp7 (lanes 2 and 3), *A. lipoferum* SpRG20a (lanes 4 and 5), and *A. amazonense* Y1 (lanes 6 and 7) were prepared from 450-ml culture samples before (lanes 2, 4, and 6) and after (lanes 3, 5, and 7) the addition of 1 mM ammonium chloride. The same extracts were used to determine the in vitro nitrogenase activity (Table 2). As a reference, purified, inactive (modified) Fe protein of *R. rubrum* was electrophoresed in lane 1.

subunits (Fig. 5). The slower-migrating subunit, resembling the modified, inactive Fe protein subunit in *R. rubrum*, appeared to have increased over the other subunit on NH_4^+ switch-off (Table 2). Before and after the addition of ammonium, two very closely migrating Fe protein subunits of *A. amazonense* Y1 were present (Fig. 5). No band migrated like the modified subunit of *R. rubrum*.

Interestingly, the nitrogenase activities in extracts of *A. brasilense* and *A. lipoferum* were less than 1/10 those in extracts of *A. amazonense* (Table 2). As predicted from this, extracts of N_2-fixing *A. brasilense* and *A. lipoferum* cultures showed an inactive subunit type of the Fe protein on SDS-PAGE, but those of *A. amazonense* did not (Fig. 5). Possibly during the time-consuming harvest and sonication of 450-ml samples, nitrogenase was partially inactivated; thus, we adopted a quick sampling and extraction method to investigate the underlying mechanism. To test for the involvement of phosphate in Fe protein inactivation, the NH_4^+ switch-off was performed with a ^{32}P-labeled, N_2-fixing culture of *A. brasilense* Sp7. "Quick extracts" were prepared either without or after the addition of 1 mM NH_4Cl. ^{32}P-labeled extracts were immunoprecipitated with antiserum against the *R. rubrum* Fe protein; this included protein A (*S. aureus* cells) to improve the yield during the washing steps. The autoradiogram of the SDS-PAGE clearly demonstrated the incorporation of ^{32}P into the slower-migrating Fe protein subunit of *A. brasilense* Sp7 on NH_4^+ switch-off. Immunoblotting experiments of quick extracts with antiserum against the *R. rubrum* Fe protein revealed a modified subunit upon NH_4^+ switch-off in *A. brasilense* and *A. lipoferum* (Fig. 6).

DISCUSSION

A rapid inhibition of nitrogen fixation by ammonium was observed in all three species of *Azospirillum*. In *A. brasilense* Sp7 and *A. lipoferum* SpBr17 and SpRG20a, the response of the nitrogenase activity to the addition of ammonium closely resembled the response in *R. rubrum* and *Rhodopseudomonas capsulata* (15, 16). However, the regulation of nitrogen fixation in *A. amazonense* Y1 was different in several ways.

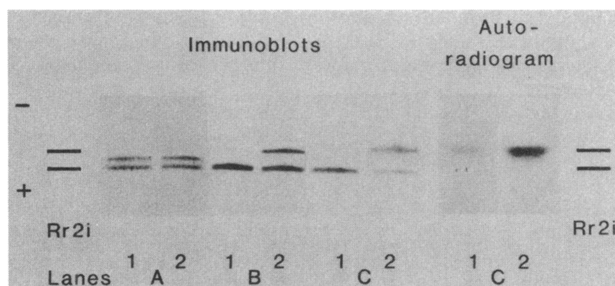

FIG. 6. Autoradiography and immunoblots with Fe protein antiserum of SDS-PAGE with quick extracts from *Azospirillum* cultures. Culture samples were harvested by filtration without (lanes 1) and 20 min after (lanes 2) the addition of 1 mM ammonium chloride. A, *A. amazonense* Y1; B, *A. lipoferum* SpRG20a, C, *A. brasilense* Sp7. Isolated modified Fe protein of *R. rubrum* (Rr2i; Fig. 5) was used as a standard to align the immunoblots and the autoradiogram. For the immunoblotting experiments, 15 μl of the quick extracts was mixed with 70 μl of SDS sample buffer. For autoradiography, extracts of a ^{32}P-labeled culture were immunoprecipitated with Fe protein antiserum before electrophoresis (see the text).

In all three species, very low concentrations of extracellular ammonium caused a switch-off of nitrogenase activity; switch-on occurred when the added ammonium appeared to be used up (Fig. 1A through C). The inhibition with 1 mM NH_4Cl was complete in *A. brasilense* and *A. lipoferum*, but only partially so in *A. amazonense*. Concentrations up to 10 mM ammonium did not completely inhibit nitrogen fixation in *A. amazonense*. This difference in effectiveness of NH_4^+ switch-off was not due to different ammonium uptake, because all three *Azospirillum* species have a high-affinity ammonium carrier under N_2-fixing conditions (12; A. Hartmann, unpublished data).

The effects of MSX (an inhibitor of GS), azaserine (an inhibitor of glutamate synthase), and glutamine on the nitrogenase activity of *Azospirillum* species suggested glutamine as a mediator for the rapid inhibition by ammonium; it has been implicated in several other species (37). The fact that mutants of *A. brasilense* deficient in GS fix nitrogen in the presence of ammonium also indicates that ammonium has to be converted to glutamine to exert its effect on nitrogenase (7). The inability of MSX to abolish the NH_4^+ switch-off in *A. amazonense* suggests a different mechanism for the short-term inhibition of nitrogen fixation by ammonium in this organism. However, MSX did not inhibit GS in *A. amazonense* cells like it did in *A. brasilense*, whereas in vitro, the GS of each organism was sensitive to MSX (Table 1). If MSX is taken up poorly by *A. amazonense*, it may be ineffective in abolishing the inhibition of nitrogenase activity by ammonium. *A. brasilense* transports MSX effectively, but certain other bacteria (e.g., *Azotobacter vinelandii*) do not (17). One must be cautious in interpreting the effects of MSX on the NH_4^+ inhibition of nitrogenase in the absence of information on the uptake of MSX. Also, MSX may block ammonium uptake itself (17, 34) and thus complicate its use in the study of switch-off.

Optimal nitrogen fixation occurs in a narrow range of low oxygen concentrations in the microaerobic *Azospirillum* species, so it is important to measure and control the dissolved-oxygen concentration during NH_4^+ switch-off experiments. The experiments performed conveniently in shaken bottles with 0.6 to 1.0 kPa of oxygen in the gas phase (Fig. 1 and 2) exhibited linear rates of acetylene reduction. However, oxygen in the gas phase and in the medium was not in equilibrium. Measurements of NH_4^+ switch-off in a chamber with a rapidly spinning magnetic bar and a probe for dissolved oxygen (13) revealed details of the nitrogenase inhibition by ammonium at different, constant dissolved-oxygen levels.

In *A. brasilense* and *A. lipoferum* it was between 15 and 20 min after the addition of ammonium before the nitrogenase was completely inhibited (Fig. 3). This suggested that nitrogenase was undergoing enzymatic inactivation. The inactivation by covalent modification of the Fe protein was corroborated by ^{32}P incorporation into the slower-migrating subunit of the Fe protein and by the appearance of two types of Fe protein subunits on SDS-PAGE, as in *R. rubrum* (Fig. 6). The activation of the inactive Fe protein appears closely related among *A. brasilense*, *A. lipoferum*, and *R. rubrum*.

A. amazonense, however, exhibited reduced rates of nitrogen fixation without a transition period after the addition of ammonium chloride (Fig. 4). Because no decrease of in vitro nitrogenase activity and no evidence for a modified subunit of the Fe protein was found in extracts of the ammonium-treated cells of *A. amazonense*, an enzymatic inactivation of nitrogenase probably is not involved. The increased inhibition by ammonium at higher, partially inhibitory oxygen concentrations also has been reported for *Azotobacter vinelandii* (19), and an ammonium-induced decrease in the membrane potential has been correlated with the rapid inhibition of nitrogen fixation in *Azotobacter vinelandii* (21). A similar mechanism may be involved in the inhibition of nitrogenase by ammonium in *A. amazonense* Y1.

The nitrogenase activities of extracts of *A. brasilense* and *A. lipoferum* were less than 10% of those in *A. amazonense*. This low activity correlated with the appearance of the modified Fe protein on SDS-PAGE in *A. brasilense* and *A. lipoferum* extracts even without specific switch-off treatment (Fig. 5). The inactivating reaction, possibly triggered by anaerobic conditions produced during cell harvest, centrifugation, suspension and sonication, apparently was dominant and thus produced a predominantly inactive Fe protein in cell-free preparations of *A. brasilense* and *A. lipoferum*. A similar response was reported for glutamate-grown *R. rubrum* (16). When the azospirilla were harvested quickly by filtration and the extracts were prepared by grinding, no modified subunit of the Fe protein was found in actively fixing cells (Fig. 6).

The nitrogenase activities of *A. amazonense* extracts were comparable to those of their whole cells and to an extract from *Azotobacter vinelandii*. An inactivation reaction for the Fe protein apparently does not operate in *A. amazonense* under the variety of conditions we tested. In all highly active extracts of *A. amazonense*, a closely migrating double band was found for the Fe protein subunits on SDS-PAGE. This migration behavior was distinct from that of *A. brasilense*, *A. lipoferum*, or *R. rubrum* (Fig. 5 and 6). In addition, two closely migrating subunit species have been found in the highly active, purified Fe protein of *A. amazonense* and *Azotobacter vinelandii* (20,33a).

In summary, although a rapidly induced inhibition of nitrogenase by ammonium chloride could be demonstrated in all three *Azospirillum* species, only in *A. brasilense* and *A. lipoferum* was a covalent modification of the Fe protein implicated in the inactivation of nitrogenase in NH_4^+ switch-off. The weak inhibition of nitrogen fixation by ammonium in *A. amazonense* Y1 is a favorable feature for a root-colonizing bacterium that has potential as a biofertilizer.

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