Posttranslational Regulatory System for Nitrogenase Activity in Azospirillum spp.

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The mechanism for "NH₄⁺ switch-off/on" of nitrogenase activity in Azospirillum brasilense and A. lipoferum was investigated. A correlation was established between the in vivo regulation of nitrogenase activity by NH₄Cl or glutamine and the reversible covalent modification of dinitrogenase reductase. Dinitrogenase reductase ADP-riboisyltransferase (DRAT) activity was detected in extracts of A. brasilense with NAD as the donor molecule. Dinitrogenase reductase-activating glycohydrolase (DRAG) activity was present in extracts of both A. brasilense and A. lipoferum. The DRAG activity in A. lipoferum was membrane associated, and it catalyzed the activation of inactive nitrogenase (by covalent modification of dinitrogenase reductase) from both A. lipoferum and Rhodospirillum rubrum. A region homologous to R. rubrum draT and draG was identified in the genomic DNA of A. brasilense as a 12-kilobase EcoRI fragment and in A. lipoferum as a 7-kilobase EcoRI fragment. It is concluded that a posttranslational regulatory system for nitrogenase activity is present in A. brasilense and A. lipoferum and that it operates via ADP-ribosylation of dinitrogenase reductase as it does in R. rubrum.

Biological nitrogen fixation is catalyzed by the nitrogenase complex, which consists of two proteins: dinitrogenase (MoFe protein) and dinitrogenase reductase (Fe protein) (21). During the reaction, dinitrogenase reductase donates electrons to dinitrogenase one at a time, with concomitant ATP hydrolysis. The reduced dinitrogenase catalyzes the reduction of N₂ to 2 NH₃ molecules and reduction of two protons to H₂ (33). This is an energy-demanding process, theoretically consuming at least 16 ATP molecules for each N₂ molecule reduced. It is not surprising that the nitrogenase system is highly regulated at both the gene expression level (8) and the posttranslational level (23, 38).

Exogenous NH₄⁺ rapidly and reversibly inhibits nitrogenase activity in whole cells of a variety of nitrogen-fixing microorganisms (23; 38 and references therein). The mechanism for NH₄⁺ switch-off/on (terminology of Zumft and Castillo (39)) has been well studied in the purple nonsulfur photosynthetic bacterium Rhodospirillum rubrum. In R. rubrum, the loss of cellular nitrogenase activity during switch-off by NH₄Cl is correlated with the covalent modification and resulting inactivation of dinitrogenase reductase (13). The covalent modification consists of the ADP-ribosylation of dinitrogenase reductase at Arg-101 (29). This reaction is catalyzed by dinitrogenase reductase ADP-riboisyltransferase (DRAT) (18, 19). Dinitrogenase reductase is composed of two identical subunits, and the ADP-ribosylated subunit migrates more slowly than the unmodified subunit during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Dinitrogenase reductase-activating glycohydrolase (DRAG) catalyzes the removal of the ADP-ribose group from the inactive dinitrogenase reductase, thereby reactivating the dinitrogenase reductase (20, 31, 32).

Genes coding for the R. rubrum DRAT and DRAG activities have been cloned and sequenced (W. P. Fitzmaurice, L. L. Saari, R. G. Lowery, P. W. Ludden, and G. P. Roberts, Mol. Gen. Genet., in press). The draT gene is transcriptionally upstream of draG, and the dra region is located next to nifH, which codes for dinitrogenase reductase, the substrate for DRAT and DRAG. Substantial evidence has been provided that this type of covalent modification also operates in Rhodobacter capsulatus (12; J. Jouanneau, C. Meyer, and P. M. Vignais, in H. Bothe, F. J. de Bruijn, and W. E. Newton, ed., Nitrogen Fixation: Hundred Years After, 1988, p. 173), Chromatium vinosum (7), and Azospirillum brasilense and Azospirillum lipoferum (10). The Azospirillum species are the only nonphotosynthetic bacteria so far demonstrated to possess this covalent modification system for nitrogenase.

Azospirillum spp. are gram-negative, vibroid, microaerobic, N₂-fixing bacteria (4). Four species have been described: A. brasilense, A. lipoferum (36), A. amazonense (24), and A. halopraeferans (30). They fix N₂ in soil and in association with the roots of economically important cereal crops, such as corn, wheat, and rice (4). It has been suggested that when Azospirillum spp. colonize roots of cereals and forage grasses, they increase yields by improving root development, increasing the rate of water and mineral uptake, and providing biologically fixed nitrogen (27). Because of their potential agronomic importance, considerable research has been focused on the azospirilla (4). A better understanding of their nitrogenase system and its regulation will be helpful in the application of the azospirilla as effective biofertilizers.

We have demonstrated that NH₄Cl reversibly inhibits nitrogenase activity in A. brasilense, A. lipoferum, and A. amazonense (10) and have suggested that NH₄⁺ regulates nitrogenase activity by covalent modification of dinitrogenase reductase in A. brasilense and A. lipoferum, but not in A. amazonense (10, 34). Here we further characterize the posttranslational regulatory system for nitrogenase activity of A. brasilense and A. lipoferum and report further evi-
Azospirillum spp. were grown in nutrient broth with trichloroacetic acid (TCA; 25 mM) and dithionite. The chloride, 30 imol of MOPS buffer was measured as the acetylene reduction rate (3). For the in vitro assay, the acetylene reduction activity of the culture was monitored by adding N$_2$-fixing cells of A. brasilense and A. lipoferum, 200 mL of culture was grown for 24 h at 30°C on minimal medium with 20 mM NH$_4$Cl, and this was used to inoculate 2 liters of minimal medium. The optimal dissolved oxygen concentration in equilibrium with 0.1 or 0.3 kPa of O$_2$, respectively, in the gas phase was kept constant with an oxystat. The pH was controlled at 6.8 with a pH-stat. Nitrogenase was derepressed for the cells is expressed as the acetylene reduction activity in vitro, for the in vitro assay, the dinitrogenase reductase activity was measured by adding the initial activity was consumed.

To assay DRAT activity, aerobically growing Azospirillum cultures (1 liter each) in minimal medium with NH$_4$Cl (10 mM) were harvested by centrifugation at 7,000 × g for 10 min. The cells were suspended in 2 mL of MOPS buffer (100 mM MOPS [pH 7.5], 1 mM ATP, 50 μM EDTA, and 1 mM dithiothreitol) and lysed by adding 2 mg of lysozyme 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. After centrifugation at 125,000 × g for 2 h at 4°C, the supernatant was used as a crude extract in the DRAT assay.

Alternatively, crude extracts were prepared anaerobically essentially as described by Hartmann et al. (10). Frozen cell paste of A. lipoferum was thawed in 2 volumes of Tris buffer (250 mM Tris [pH 8.5] containing 4 mM dithionite and 1 mM dithiothreitol) and incubated with lysozyme (1 mg/mL) for 30 min on ice. After sonication and centrifugation as described above, the supernatant (supernatant in Table 2) was used as a crude extract to assay DRAT activity and for making nitrogense complex preparations. The nitrogenase complex (inactive nitrogense mix) is the substrate for the DRAT assay. This nitrogenase complex contains active dinitrogenase and ADP-ribosylated dinitrogenase reductase (dark-initiated for R. rubrum [13] and anaerobically initiated for A. lipoferum [9]).

To determine the location of the DRAG activity, the pellet fraction was suspended in low-concentration buffer (25 mM Tris-acetate, pH 7.8) and centrifuged again at 125,000 × g for 3 h. The resulting supernatant was the wash fraction, and the pellet was suspended in high-salt buffer (0.5 M NaCl in 25 mM Tris-acetate, pH 7.8). The salt wash fraction I was collected after centrifugation as above. The salt-washing step was repeated once to obtain the second fraction of salt wash (II). The membrane portion of the final pellet was suspended in 25 mM Tris buffer as the final fraction. All these fractions were stored in liquid nitrogen until assayed.

To prepare the inactivated nitrogense complex of A. lipoferum and R. rubrum (32), crude extracts were diluted (50 mM Tris-acetate, final concentration) and loaded onto an anaerobic DEAE-52 cellulose column (2.5 by 10 cm) and washed with 25 mM Tris buffer (pH 7.8) containing 10 mM NaCl. Both dinitrogenase and dinitrogenase reductase were eluted in the same fraction by the Tris buffer (25 mM, pH 7.8) containing 450 mM NaCl, and this fraction was desalted on a G-25 Sepharose column. The inactive nitrogense mix was used as the substrate for the DRAG assay.

The flowthrough fraction recovered while loading the crude extracts of A. lipoferum from the DEAE-52 column above was collected on a hydroxypatite column. After the column was washed with 25 mM Tris buffer (pH 7.8), the DRAG activity was eluted with 100 mM potassium phosphate buffer, pH 7.8 (hydroxypatite fraction in Table 2).

**DRAT assay.** DRAT activity was assayed under anaerobic conditions by determination of the radioactivity of [α-32P]ADP-ribose incorporated into dinitrogenase reductase with NAD as the donor molecule (19). The reaction mixture contained 0.25 mM exogenous NAD, 0.6 μCi of [α-32P]NAD, 0.25 mM ADP, 5 mM MgCl$_2$, 100 μg of purified active dinitrogenase reductase (when present), and the DRAT fraction in MOPS buffer (100 mM, pH 7.0), in a total volume of 50 μl. The reaction was started by adding active dinitrogenase reductase and incubating at 30°C for 50 min. After the reaction, 20 μl of the reaction mixture was precipitated in 5% TCA and suspended in SDS-PAGE buffer for gel electrophoresis and autoradiography. The remaining mixture was precipitated with TCA, and proteins were collected on nitrocel-
lulose filters. The radioactivity incorporated was measured by liquid scintillation counting. The specific incorporation of $^{32}$P into dinitrogen reductase was confirmed by immunoblotting analysis and autoradiography. Dinitrogen reductase from Klebsiella pneumoniae was used as the substrate in the DRAT assays. The activity was expressed as counts per minute in TCA-precipitated fractions.

NAD degradation activity of Azospirillum crude extracts was monitored by incubating the extracts (15 μg) with ADP (0.25 mM), MgCl$_2$ (5.0 mM), and NAD (0.25 mM; 0.5 μCi of $^{[32]$P$]NAD}$ for 15 min anaerobically at 30°C. A sample of the reaction mixture was separated by polyethyleneimine-cellulose thin-layer chromatography (TLC) sheets at 30°C. The autoradiogram was developed from the TLC sheets.

**DRAG assay.** DRAG activity was estimated by coupling the activation of the inactive dinitrogen reductase to the acetylene reduction assay for nitrogenase activity (32). In the absence of the DRAG enzyme, the inactive nitrogenase mixture showed low background activity, and this background activity was subtracted from the activity values obtained with DRAG present. The acetylene reduction rate is calculated on the protein of the DRAG fraction.

**SDS-PAGE and enzyme-linked immunoblotting.** An SDS-PAGE system was used essentially as described by Laemmli (15) or as modified by Kanemoto and Ludden (13) to provide satisfactory resolution of the dinitrogen reductase subunits in a 10% acrylamide gel (see figure legends). Calculations: $\% T = \{\text{[acrylamide (g) + bisacrylamide (g)}/\text{volume (ml)}\} \times 100$, $\% C = \{\text{[bisacrylamide (g)}/[\text{acrylamide (g) + bisacrylamide (g)}]\} \times 100$. The enzyme-linked immunoblotting procedures of Towbin et al. (37) as modified by Hartmann et al. (10) were followed. Electrophoresed proteins in SDS-PAGE were electrophoretically transferred to nitrocellulose membranes and incubated with polyclonal antibody; cross-reacting material was visualized with horseradish peroxidase conjugated to goat anti-rabbit immunoglobulin G. After staining, the protein bands on the wet nitrocellulose membranes were scanned with a Zeineh soft-laser densitometer.

**Protein assay.** Protein concentrations were determined with the micro-biuret method of GoA (6) with bovine serum albumin as the standard.

**DNA preparation.** The total DNA of each Azospirillum spp. and *R. rubrum* was isolated by the procedures of Maniatis et al. (25). For making hybridization probes, plasmid DNA was isolated by the alkaline extraction procedure of Birnboim and Doly (2) and digested with appropriate restriction enzymes (see below). DNA fragments were separated on agarose gels by electrophoresis, and the specific bands containing *draT* or *draG* were excised and purified from the gel with a GeneClean Kit (Bio 101, La Jolla, Calif.) according to the manufacturer’s specifications.

**32P-labeling of DNA probes and Southern hybridization.** The restriction fragment probes of *draT* or *draG* (0.6 kilobase [kb] BamHI-SacII fragment and 0.55-kb EcoRI-HindIII fragment of pWPF102, respectively; Fitzmaurice et al., in press) were labeled with $^{32}$P by the oligo-labeling procedure of Feinberg and Vogelstein (5). The incorporation of $^{32}$P into DNA probes was verified by TLC.

Southern hybridization was performed essentially as described by Maniatis et al. (25) with some modifications. After hybridization at 42°C overnight, the following conditions were used for washing: two cycles of 2× SSC at 30°C; 2× SSC with 0.1% SDS at 42°C for 30 min each; and two cycles of 2× SSC at room temperature for 30 min each. The stringent washing conditions were: two cycles of 2× SSC at room temperature, 5 min each; two cycles of 2× SSC with 1% SDS at 65°C, 30 min each; and two cycles of 0.1× SSC at room temperature, 30 min each.

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

**RESULTS**

**Correlation between in vivo regulation of nitrogenase activity and reversible modification of dinitrogen reductase.** In *A. brasilense* and *A. lipoferum*, the addition of NH$_4$Cl or glutamine to derepressed cells gradually and completely inhibited nitrogenase activity (10). The reversibility of the inhibition could be observed over a short time course when low concentrations of inhibitors were used, e.g., 0.2 mM NH$_4$Cl (Fig. 1A) (10). To monitor dinitrogen reductase modification during the process of reversible inhibition of nitrogenase activity in vivo, cell samples were collected rapidly (30 s) on filters and frozen in liquid nitrogen to quench cellular metabolism. Acetylene reduction rates of whole cells and the dinitrogen reductase electrophoretic pattern were monitored at the same time. Figure 1 shows the switch-off and switch-on of nitrogenase activity in the presence and absence of NH$_4$Cl in *A. brasilense* Sp7. Before NH$_4$Cl treatment, nitrogenase was active and dinitrogen reductase appeared as a single type of subunit, indicative of the active form (Fig. 1B). At 10 min after NH$_4$Cl (0.2 mM) addition, whole-cell nitrogenase activity was gradually inhibited (Fig. 1A) and a second type of subunit of dinitrogen reductase increased markedly concomitantly (Fig. 1B). This subunit moved electrophoretically slightly above the unmodified subunit. The intensity of the modified subunit was similar to that of the unmodified form, suggesting a 1-to-1 ratio. After 45 min of NH$_4$Cl treatment, nitrogenase activity recovered, apparently because of exhaustion of the exogenous NH$_4^+$; at the same time, the intensity of the modified bands decreased. It is the modified subunit of dinitrogen reductase that becomes radiolabeled when treated with NH$_4$Cl in vivo in the presence of $^{32}$P in the medium (10). For *A. lipoferum*, inhibition of nitrogenase by NH$_4$Cl in vivo was also correlated with the covalent modification of dinitrogen reductase (data not shown).

Glutamine, like NH$_4$Cl, also causes inhibition of nitrogenase activity in vivo in *Azospirillum* spp. (10). Figure 2 shows how the subunit pattern of dinitrogen reductase changed during the process of glutamine inhibition. Before glutamine addition, there was only one type of detectable subunit for dinitrogen reductase. The inhibition of nitrogenase activity in vivo by 0.5 mM glutamine (5 and 10 min, data not shown) was also accompanied by the appearance of the slower-migrating bands (lanes 2 and 3) of dinitrogen reductase; these comigrated with the ADP-ribosylated subunit of dinitrogen reductase from *R. rubrum* (upper band of lane 7). When the nitrogenase activity resumed after the depletion of added glutamine (55 min after 0.15 mM glutamine was added), the modified subunit of dinitrogen reductase disappeared from the gel (lane 4).

Addition of methionine sulfoximine (MSX), an inhibitor of glutamine synthetase, prevents the inhibitory effect of NH$_4^+$ on nitrogenase (10); it also reverses NH$_4^+$ inhibition if added after NH$_4$Cl. Moreover, we have observed that MSX re-
verses both the NH₄⁺ inhibition and the modification of dinitrogen reductase. Azaserine inhibits the NH₄⁺ assimilation pathway primarily by blocking the glutamine oxoglu-tarate aminotransferase reaction. The inhibition by azaserine in turn probably increases the intracellular glutamine concentra-tion (14). The addition of 1 mM azaserine to A. lipoferum cells derepressed for nitrogenase gradually decreased their acetylene reduction rate, and the modified subunit of dinitrogenase reductase appeared simultaneously (lanes 5 and 6, Fig. 2).

**DRAT activity.** Table 1 and Fig. 3 show NAD-dependent DRAT activity in crude extracts of A. brasilense. Azospirillum cultures were grown in the minimal medium with excess NH₄⁺. The dinitrogen reductase of K. pneumoniae was used as the substrate, because it gives high activity in the in vitro assay for R. rubrum DRAT (18).

The crude extract of A. brasilense, in the presence of MgCl₂ and ADP, catalyzed the incorporation of ³²P from [³²P]NAD into the TCA-precipitable fractions (Table 1). This DRAT assay is dependent on the presence of dinitrogen reductase, and in its absence only background labeling was recorded. Inclusion of the partially purified DRAT in the reaction mixture caused a dramatic increase in radioactivity retained on the filter. The ³²P in the TCA-precipitated fractions was specifically incorporated into the dinitrogen reductase of the reaction mixture (Fig. 3). When proteins in a sample of reaction mixture (Table 1) were separated on SDS-PAGE, only one band was radiolabeled (lane 2, Fig. 3). This band corresponded to the position of dinitrogen reductase from K. pneumoniae, which was revealed by cross-reaction with antiserum produced against dinitrogen reductase. The purified DRAT alone or DRAT plus extracts of A. brasilense gave high activity (samples 4 and 5, Table 1) as a positive control and showed an intensely radiolabeled band on the autoradiogram (lanes 4 and 5), which comigrated with the band from A. brasilense extract. Lanes 1 and 3 showed that no proteins were labeled if

**FIG. 1.** Reversible modification of dinitrogenase reductase during NH₄⁺ switch-off/on of nitrogenase activity in A. brasilense Sp7. (A) Nitrogenase activity in vivo measured in a controlled pO₂ chamber. After 15 min (arrow), 0.2 mM NH₄Cl was added. (B) Scannings of immunoblots with antiserum against R. rubrum dinitrogenase reductase. Culture samples (5 ml) were withdrawn during the course of the NH₄⁺ switch-off/on experiment (panel A), collected rapidly on microfiber filters, and frozen. The extracts were prepared by grinding the filter in anaerobic buffer and analyzed with SDS-PAGE (10% T, 0.58% C) followed by immunoblotting. Samples were collected before (+) or 10 min (+ inside circle; switch-off phase) or 45 min (+ inside two circles; switch-on phase) after the addition of 0.2 mM NH₄Cl. Peak i. Covalently modified subunit of dinitrogen reductase; peak a. unmodified form.

**FIG. 2.** Immunoblots of quick extracts from A. lipoferum during glutamine switch-off/on with antiserum against R. rubrum dinitroge-nase reductase. As indicated in the legend to Fig. 1B, samples were collected before (lane 1) or 5 or 10 min after (lanes 2 and 3; switch-off of nitrogenase activity in vivo) addition of 0.5 mM glutamine or 35 min after the addition of 0.15 mM glutamine (lane 4; switch-on of nitrogenase activity). Lanes 5 and 6 represent samples from cells treated with 1 mM azaserine in place of glutamine for 10 and 20 min, respectively. Lane 7 is R. rubrum ADP-ribosylated dinitrogen reductase as a control.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Addition to reaction mix*</th>
<th>K₃₂ added (µg)</th>
<th>DRAT activity (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A. brasilense extract</td>
<td>None</td>
<td>609</td>
</tr>
<tr>
<td>2</td>
<td>A. brasilense extract</td>
<td>100</td>
<td>21,464</td>
</tr>
<tr>
<td>3</td>
<td>A. brasilense extract + DRAT</td>
<td>None</td>
<td>506</td>
</tr>
<tr>
<td>4</td>
<td>A. brasilense extract + DRAT</td>
<td>100</td>
<td>61,663</td>
</tr>
<tr>
<td>5</td>
<td>DRAT only</td>
<td>100</td>
<td>64,821</td>
</tr>
<tr>
<td>6</td>
<td>None (negative control)</td>
<td>100</td>
<td>418</td>
</tr>
</tbody>
</table>

*Crude extract from A. brasilense (750 µg) was used. DRAT was partially purified from R. rubrum.

K₃₂: Dinitrogenase reductase from K. pneumoniae.

DRAT activity was determined by the amount of ³²P transferred from [³²P]NAD to the TCA-precipitable fraction of the reaction mixture as measured by a liquid scintillation counter.
dinitrogenase reductase was omitted from the reaction system.

The extracts of *A. lipoferum* did not give any appreciable amount of DRAT activity. However, the reaction mixture that included purified DRAT also showed only background counts, suggesting that crude extracts of *A. lipoferum* may contain a high level of an NAD-degrading activity. To test this possibility, [\(^{32}\)P]NAD was incubated in the extracts of *A. brasilense* and *A. lipoferum* for 15 min, and the products were subjected to TLC and autoradiography. NAD was completely degraded by *A. lipoferum* extracts, but not by extracts from *A. brasilense* (data not shown).

**DRAG activity.** In *R. rubrum*, the chromatophore membrane-associated DRAG activity is responsible for the in vitro activation of inactive dinitrogenase reductase by the removal of the ADP-ribose group (20, 30, 32). DRAG has been purified to homogeneity (32). *A. brasilense* Sp7 has a three-component nitrogenase system (22): dinitrogenase, dinitrogenase reductase, and an activating factor (DRAG). DRAG has been isolated from *A. brasilense* and can reactivate the inactive nitrogenase of *A. brasilense* (17, 22). The rate of the reactivation is increased by Mn(II). DRAG of *R. rubrum* can substitute for DRAG of *A. brasilense* to reactivate the inactive dinitrogenase reductase from *A. brasilense*.

Table 2 shows the activation of inactive nitrogenase by DRAG fractions of *A. lipoferum* SpBr17 and indicates that the DRAG activity of *A. lipoferum* is associated with membranes, as it is in *A. brasilense* and *R. rubrum* (17, 20, 22, 32). The DRAG activity could be solubilized with Tris buffer (25 mM, pH 7.8) containing 0.5 M NaCl; after repeated washes with the buffer, some activity still remained on the membranes. The supernatant of the crude extract from *A. lipoferum* contained relatively high DRAG activity when suspended in 250 mM Tris-acetate (pH 8.5); the high-ionic-strength buffer may have aided in dissociating DRAG protein from the membranes.

The DRAG of *A. lipoferum* did not bind to a DE-52 column; it could be concentrated on a column of hydroxyapatite and eluted with 100 mM potassium phosphate, pH 7.8.

**Table 2.** Activation of inactive nitrogenase by DRAG fractions of *A. lipoferum* SpBr17

<table>
<thead>
<tr>
<th>DRAG fraction</th>
<th>Inactive nitrogenase mix ((\mu)g)</th>
<th>Nitrogenase activity (nmol of (\text{C}_2\text{H}_4/\text{min per mg})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer wash</td>
<td>500</td>
<td>0.0</td>
</tr>
<tr>
<td>Salt wash I</td>
<td>500</td>
<td>15.0</td>
</tr>
<tr>
<td>Salt wash II</td>
<td>500</td>
<td>15.5</td>
</tr>
<tr>
<td>Final pellet</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>No additiona</td>
<td>500</td>
<td>0.0</td>
</tr>
<tr>
<td>Supernatant</td>
<td>500</td>
<td>34.7</td>
</tr>
<tr>
<td>Hydroxyapatite (100 mM (\text{PO}_4^{3-}))</td>
<td>500</td>
<td>108.0</td>
</tr>
</tbody>
</table>

\(a\) Partially purified nitrogenase with inactive dinitrogenase reductase complex from *R. rubrum* (see Materials and Methods).

\(b\) No DRAG fraction was added to the reaction mixture; this is a negative control.

of the fractions with anti-*R. rubrum* DRAG serum (data not shown).

**Detection of draT- and draG-homologous sequences.** In further characterization of the posttranslational regulatory system for the nitrogenase of *Azospirillum* spp., we investigated its sequence homologies with the *draT* and *draG* genes of *R. rubrum*. Figure 4 shows a Southern hybridization of *Azospirillum* spp. DNA with a \(32^P\)-labeled *draT* probe from *R. rubrum*. Under low-stringency conditions (see Materials and Methods), a band indicating strong hybridization (12-kb *EcoRI* fragment) appeared in genomic DNA of *A. brasilense* (lane 2) and a band of about 7 kb appeared for *A. lipoferum* (lane 3). No signal was obvious for *A. amazonense* (lane 1), an organism for which no covalent modification system for dinitrogenase reductase has been demonstrated. The genomic DNA of *R. rubrum* showed the 2.1-kb *EcoRI* fragment containing the *draT* region (lane 5). The nature of the upper band remains unclear. It was also observed that the intragenic *draG* probe and the *draT* probe hybridized to form bands in essentially the same positions with *A. brasilense* and *A. lipoferum* and exhibited no signal for *A. amazonense*; under high-stringency conditions with the *draG* probe from

**FIG. 4.** Southern hybridization of genomic DNA with a *draT* probe from *R. rubrum*. Total DNA from *A. amazonense* (lane 1), *A. brasilense* (lane 2), *A. lipoferum* (lane 3), and *R. rubrum* (lane 5) was digested with *EcoRI* and hybridized to a \(32^P\)-labeled *draT* probe from *R. rubrum*. (A) Ethidium bromide-stained DNA on the agarose gel; (B) autoradiogram. Bacteriophage lambda DNA cut with *HindIII* provided size markers and a negative control (lane 4).
R. rubrum, only A. brasilense showed a hybridization band (data not shown). These results indicate that the draT and draG of R. rubrum have greater homology with those of A. brasilense than with those of A. lipoferon.

DISCUSSION

The in vivo regulation of nitrogenase activity by NH$_4$Cl or glutamine has been correlated with the reversible covalent modification of dinitrogenase reductase. Apparently this is the first demonstration that regulation of nitrogenase activity by covalent modification in a nonphotosynthetic bacterium is reversible. The following lines of evidence suggest that covalent modification in the presence of NH$_4^+$ or glutamine results from ADP-ribosylation of dinitrogenase reductase. (i) The modified subunit of dinitrogenase reductase from A. brasilense and A. lipoferon comigrated with that of the ADP-ribosylated subunit of dinitrogenase reductase from R. rubrum, and the modified subunit was $^{32}$P-labeled in vivo upon NH$_4^+$ treatment. (ii) In the in vitro assay of DRAT activity from A. brasilense, the enzyme utilized NAD as the donor molecule and dinitrogenase reductase as a substrate. (iii) The DRAG activities of A. brasilense and A. lipoferon were functionally interchangeable with the DRAG activity of R. rubrum when ADP-ribosylated dinitrogenase reductase was the substrate. (iv) draT- and draG-homologous regions were both detected in genomic DNA of A. brasilense and A. lipoferon, but not in the genomic DNA of A. amazonense, which lacks the system for control of nitrogenase by covalent modification. This fact provides a further correlation between the regulation of nitrogenase activity by covalent modification and the existence of the genes coding for DRAT and DRAG. We recently showed these regions to be functional in allowing the expression of DRAT and DRAG activity (data not shown). All data lead to the reasonable conclusion that ADP-ribosylation of dinitrogenase reductase affects posttranslational regulation of nitrogenase activity in A. brasilense and A. lipoferon.

In A. brasilense, NH$_4^+$ is assimilated by glutamine synthetase. Glutamine then serves as the nitrogen donor for the reductive amination of oxoglutarate to glutamate (28). The application of inhibitors of nitrogen metabolism revealed the importance of glutamine to the NH$_4^+$ switch-off (10, 35). MSX, an inhibitor of glutamine synthetase, prevented the inhibition of nitrogenase activity by NH$_4^+$. When MSX was added after the NH$_4^+$ treatment, the NH$_4^+$ inhibition was reversed, and this was accompanied by the demodification of dinitrogenase reductase. Azaserine, an inhibitor of glutamine oxoglutarate aminotransferase, strongly inhibited nitrogenase activity by itself, perhaps by increasing the cellular glutamine level. Azaserine inhibited nitrogenase activity in vivo, and this was accompanied by modification of dinitrogenase reductase. Glutamine, like NH$_4^+$, reversibly regulates nitrogenase activity by inducing covalent modification of dinitrogenase reductase (Fig. 2). These data support the idea that glutamine and/or a derivative of it plays an essential role in mediating the ammonium switch-off of nitrogenase, as implicated in several other systems (11, 26, 35). However, Li et al. (16) and Kanemoto and Ludden (14) thoroughly analyzed the changes in cellular glutamine levels under various switch-off conditions in R. rubrum. They found no consistent correlation between the level of glutamine (or any other amino acid) and inhibition of nitrogenase activity. These data did not rule out the possibility that glutamine (and/or a derivative) plays a crucial role in mediating the ammonium switch-off of nitrogenase activity, although it may not act as a universal signal.

Biological nitrogen fixation is an energy-demanding process. The presence of the posttranslational regulatory system for nitrogenase activity in A. brasilense and A. lipoferon should benefit survival in soil or in association with plant roots. This system may have some disadvantages in an agricultural inoculant, as a transient increase in combined nitrogen via fertilization would shut off nitrogen fixation.

It is particularly interesting that within the genus Azospirillum, NH$_4^+$ switches off nitrogenase activity by ADP-ribosylation of dinitrogenase reductase in A. brasilense and A. lipoferon but not in A. amazonense. This raises intriguing questions about the evolution of the ADP-ribosylation system for regulation of nitrogenase.

Posttranslational regulation of nitrogenase by covalent modification of dinitrogenase reductase through ADP-ribosylation has been described earlier for certain photosynthetic bacteria, and the present work demonstrates that a similar control system can function in nonphotosynthetic bacteria.

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LITERATURE CITED