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Posttranslational Regulation of Nitrogenase Activity by Anaerobiosis and Ammonium in *Azospirillum brasilense*

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In the microaerophilic diazotroph *Azospirillum brasilense*, the addition of fixed nitrogen or a shift to anaerobic conditions leads to a rapid loss of nitrogenase activity due to ADP-ribosylation of dinitrogenase reductase. The product of *draT* (DRAT) is shown to be necessary for this modification, and the product of *draG* (DRAG) is shown to be necessary for the removal of the modification upon removal of the stimulus. DRAG and DRAT are themselves subject to posttranslational regulation, and this report identifies features of that regulation. We demonstrate that the activation of DRAT in response to an anaerobic shift is transient but that the duration of DRAT activation in response to added NH_4^+ varies with the NH_4^+ concentration. In contrast, DRAG appears to be continuously active under conditions favoring nitrogen fixation. Thus, the activities of DRAG and DRAT are not always coordinately regulated. Finally, our experiments suggest the existence of a temporary period of futile cycling during which DRAT and DRAG are simultaneously adding and removing ADP-ribose from dinitrogenase reductase, immediately following the addition of a negative stimulus.

Biological nitrogen fixation is a very energy-demanding process and therefore is regulated tightly. Transcriptional regulation of *nif* gene expression, which exists in all nitrogen-fixing bacteria studied, has been characterized best in *Klebsiella pneumoniae* (26). Posttranslational regulation of nitrogenase activity, which also has been termed "switch-off" (34) or short-term inhibition (11), has been found in several diverse nitrogen-fixing bacteria (17). This regulation has been described best in *Rhodospirillum rubrum*, in which it involves reversible mono-ADP-ribosylation of dinitrogenase reductase in response to the addition of fixed nitrogen, shift from light to darkness, and other stimuli. Two enzymes have been found that perform this regulation. Dinitrogenase reductase ADP-ribosyl transferase (DRAT, the gene product of *draT*) catalyzes the transfer of the ADP-ribose from NAD to the Arg-101 residue of one subunit of the dinitrogenase reductase dimer of *R. rubrum* and thus inactivates the enzyme. Dinitrogenase reductase-activating glycohydrolase (DRAG, the gene product of *draG*) can remove the ADP-ribose from the covalently modified enzyme and restore its activity (17).

Azospirillum spp. are gram-negative, microaerobic, nitrogen-fixing bacteria associated with the roots of many economically important crops and grasses (4). Five species of this genus have been described (10, 18, 27, 29), and posttranslational regulation of nitrogenase activity by NH_4^+ has been examined in both *A. brasilense* and *A. lipoferum* (5, 7). In each case, the regulation results from reversible modification of dinitrogenase reductase as seen in *R. rubrum*. The *draTG* genes have been cloned, sequenced, and mutated in *A. brasilense*, confirming their function in vivo (33).

A. brasilense and *A. lipoferum* can perform and regulate nitrogen fixation only in a narrow range of low O_2 concentrations (0.3 to 0.8 kPa) (1, 19). Following a shift to an anaerobic condition, *A. brasilense* and *A. lipoferum* undergo ADP-ribosylation of dinitrogenase reductase and lose their nitrogenase activity (6). O_2, at concentrations of about 2% (2 kPa), can completely inhibit nitrogenase activity. Because this does not lead to ADP-ribosylation of dinitrogenase reductase (6), it suggests the involvement of a mechanism other than that of the DRAT-DRAG system.

Although the general role of DRAT and DRAG in the regulation of nitrogenase activity has been characterized, the mechanisms that regulate their effects are still unknown for any organism. It appears, however, that both DRAT and DRAG themselves are subject to posttranslational regulation. Regulation of DRAG activity in *R. rubrum* was established by the following pulse-chase experiment (8). In NH_4^+-treated *R. rubrum* cells, the turnover of the ^{32}P-labelled ADP-ribose group on dinitrogenase reductase was much slower than that of the total phosphate pool. This indicated that there was no DRAG activity under these conditions, although the protein itself was present. DRAT activity also is regulated, because derepressed DraG$^-$ mutants of *R. rubrum* and *A. brasilense* possess active dinitrogenase reductase (13, 33). As these cells have been shown to contain DRAT, it must be inactive under these conditions. Whereas candidates such as energy charge, pyridine nucleotide, and amino acids pools have been analyzed, no small molecules or protein factors have been identified in this regulation (8, 9, 12, 21, 23).

In this paper, we report switch-off of nitrogenase activity in *A. brasilense* in response to anaerobiosis and NH_4^+; they both involve the DRAT-DRAG system. We also provide new information on the specific regulation of the DRAG and DRAT proteins themselves under different physiological conditions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *A. brasilense* Sp7 (ATCC 29145), also termed UB1, is the wild-type strain obtained from the American Type Culture Collection, Rockville, Md. Two derivative strains, UB3 (*draT*, lacking DRAT) and UB4 (*draG*, lacking DRAG), have been described previously (33).

A. brasilense strains were grown in LD liquid medium (1% tryptone, 0.5% yeast extract, 0.25% NaCl) and then on LD solid medium. To obtain N_2-fixing cells, cultures grown on LD

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solid medium were used to inoculate 50 ml of NfbHP-glutamate medium (24) in a 250-ml flask, which then was incubated on a orbital shaker with a speed of 100 rpm in a 1-in.-diameter circle. This treatment caused the derepression of nitrogenase as described previously (33).

Assay for nitrogenase activity. Nitrogenase activity of whole cells was measured by their acetylene reduction rate as follows: a 1-ml sample of culture was injected into a stoppered 9-ml vial containing 20% air, 10% acetylene, and 70% argon and incubated on a reciprocal shaker at a speed of 160 1-in. excursions per min for a short time (0.5, 1, or 2 min, as indicated). One half milliliter of 4 N NaOH was injected to stop the reaction, and the ethylene produced was measured by gas chromatography (3).

Anaerobic and NH_4^+ treatments. Cells were treated anaerobically as follows: 45 ml of derepressed cells was transferred into a rubber-stoppered 120-ml bottle and made anaerobic on a manifold by evacuation and flushing with argon at least three times. One milliliter of the cells was removed anaerobically via syringe and injected into an assay vial, and acetylene reduction was measured under microaerobic conditions as described above. Cells were treated with NH_4^+ by direct addition of a small volume (usually 50 μl) of NH_4Cl to derepressed cells; anaerobic NH_4Cl solution was added to anaerobically treated cells.

TCA precipitation to prepare protein samples. To study modification of dinitrogenase reductase during switch-off and to minimize any artifactual change in the modification state during sample manipulation, trichloroacetic acid (TCA) precipitation was used to extract proteins quickly from *A. brasilense*. A 1-ml sample of the cells was mixed in a Vortex mixer with 100 μl of TCA solution (1 g of TCA per ml) in a 1.5-ml Eppendorf tube, and the mixture was cooled on ice for 10 min. After centrifugation for 30 s at 16,000 × *g*, the supernatant was removed completely, and the pellets were air-dried for 10 min before 200 μl of sodium dodecyl sulfate (SDS)-Tris buffer, containing 120 mM Tris (pH 6.8), 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 0.001% (wt/vol) bromphenol blue, 4% (vol/vol) 2-mercaptoethanol (fresh), was added to suspend all pellets. One microliter of 1 N NaOH was added to neutralize the acid, as indicated by the recovery of the blue color of the tracking dye. After centrifugation for 10 min at 14,000 rpm, the supernatant was removed to a clean Eppendorf tube and stored at −80°C for SDS-polyacrylamide gel electrophoresis (PAGE). TCA quickly denatures the proteins and stops the reaction, and it has little effect on the stability of the ADP-ribosylated group of dinitrogenase reductase.

An alternative method for quick extraction by boiling samples in SDS-Tris buffer (as described above) for 2 min was unsatisfactory, because it effected incomplete cell breakage and some loss of the ADP-ribosylated dinitrogenase reductase was incurred.

SDS-PAGE and immunoblotting of dinitrogenase reductase. Protein samples prepared by TCA precipitation were examined by SDS-PAGE with low cross-linker gels (ratio of acrylamide to bisacrylamide, 172/1) to obtain better resolution of the dinitrogenase reductase subunits; ADP-ribosylation slows the migration of the modified subunit, allowing the quantification of both modified and unmodified species (8). To avoid loss of the ADP-ribose from the protein, samples were not boiled before loading. The enzyme-linked immunoblotting procedure of Towbin et al. (30) as modified by Hartmann et al. (7) was used. Proteins from SDS-PAGE were electrophoretically transferred onto a nitrocellulose membrane, incubated with polyclonal antibody against *Azotobacter vinelandii* dinitrogenase reductase, and then visualized with horseradish peroxidase. After color development, the protein bands on the membrane were quantitated by scanning with a Zeineh Soft-Lasar Densitometer (model SL-504-XL; Biomed Instruments, Inc. Fullerton, Calif.).

Assuming that fully active dinitrogenase reductase appears as a single subunit and the completely inactive form appears as two subunits (upper and lower, with a ratio of 1:1), the percentage of active dinitrogenase reductase dimer was calculated as [(lower proteins − upper proteins)/(lower proteins + upper proteins)] × 100.

RESULTS AND DISCUSSION

In vivo assays and growth conditions. Throughout this study, we measured nitrogenase activity and the degree of modification of dinitrogenase reductase because we cannot directly assay the in vivo activities of DRAG and DRAT. Loss of nitrogenase activity and concomitant modification are indicative of DRAT activity, while restoration of nitrogenase activity and demodification are indicative of DRAG activity. For consistency, we will employ the term "switch-on conditions" to refer to those growth conditions appropriate for the derepression of the *nif* genes and the activity of nitrogenase.

Anaerobic switch-off of nitrogenase activity in *A. brasilense*. *A. brasilense* is a microaerobic N_2-fixing bacterium that requires O_2 as an electron acceptor to produce energy for growth and nitrogen fixation. When cells are shifted from a microaerobic condition to an anaerobic condition, *A. brasilense* loses its nitrogenase activity, presumably because of energy depletion or regulation of nitrogenase activity (6). To distinguish between these two, the nitrogenase activity and modification of dinitrogenase reductase were studied during microaerobic-anaerobic-microaerobic shifts in various *A. brasilense* strains. Because some O_2 must be present in the assay to support nitrogenase activity, the assays were performed under microaerobic conditions. The results are shown in Fig. 1.

In response to anaerobiosis, UB1 (wild type) showed a decline in nitrogenase activity to a residual level of 65%, followed eventually by complete recovery when the cells were returned to microaerobic conditions. The failure of strain UB3 (*draT*, lacking DRAT) to show any loss of nitrogenase activity demonstrates that DRAT is essential for this regulation. The response of strain UB4 (*draG*, lacking DRAG) to the same treatment differed from that of the wild type in two ways: the cells failed to recover nitrogenase activity upon return to microaerobic conditions, and a lower level of residual nitrogenase activity was established under anaerobic conditions. The failure of UB4 (*draG*) to recover reflects the necessity of DRAG for that recovery. These results are in general agreement with those seen previously for this organism's response to NH_4^+ (33).

The observation that strain UB1 (wild type) displays a higher residual nitrogenase activity following an anaerobic shift than does UB4 (*draG*) is consistent with reactivation of dinitrogenase reductase occurring in UB1 during the assay itself, and this model is supported by the following two lines of evidence. First, longer assay times resulted in the detection of higher nitrogenase activity. For example, following a shift of UB1 to anaerobic conditions, a 30-s assay gave a residual nitrogenase activity of 65% (relative to the initial activity), a 1-min assay gave a residual activity of 85%, and a 2-min assay gave a residual activity of nearly 100%. In contrast, different assay times had no effect on the results with UB3 (*draT*) and UB4 (*draG*), either because they lack the ability to modify dinitro-

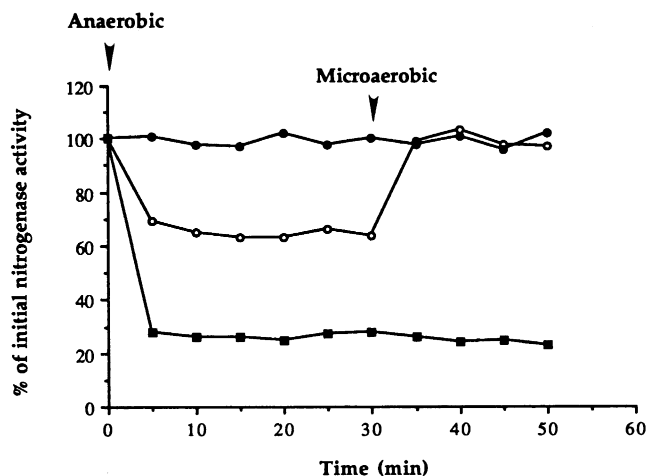

FIG. 1. Regulation of nitrogenase activity by anaerobiosis. *A. brasilense* strains UB1 (wild type), UB3 (*draT*), and UB4 (*draG*) were derepressed for *nif* expression in NfbHP-glutamate medium. At time zero, cells were made anaerobic (this takes about 5 min) and at 30 min were returned to a microaerobic condition. At the times indicated, 1-ml portions of the cells were withdrawn anaerobically and assayed for nitrogenase activity under microaerobic conditions for 0.5 min for UB1 (○) and UB3 (●) and for 2 min for UB4 (■). Initial nitrogenase activities (100%) in UB1, UB3, and UB4 were, respectively, about 1,550, 1,400, and 800 nmol of ethylene produce per h/ml of cells at an optical density at 600 nm of 5.0. Each point represents an average of at least three replicate runs.

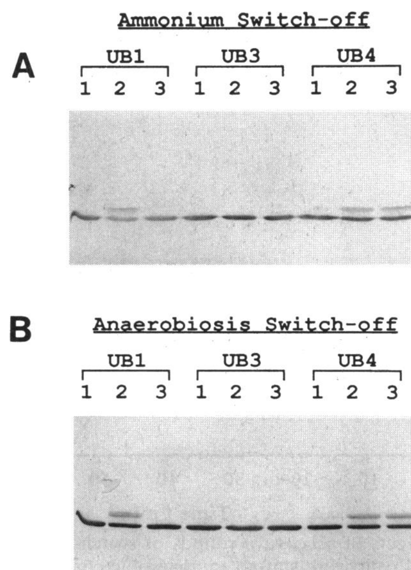

FIG. 2. ADP-ribosylation of dinitrogenase reductase in response to NH_4^+ and anaerobiosis. As described in Materials and Methods, active dinitrogenase reductase migrates as a single band, while inactive dinitrogenase reductase migrates as two bands, with the upper band representing the modified subunit. (A) Immunoblot of dinitrogenase reductase of strains UB1 (wild type), UB3 (*draT*), and UB4 (*draG*), during NH_4^+ switch-off. Crude extracts were prepared from the cells before NH_4^+ addition (lanes 1), 5 min after NH_4Cl addition (250 μM, final concentration) (lanes 2), and 15 min after NH_4Cl addition (lanes 3). (B) Immunoblot of dinitrogenase reductase of *A. brasilense* UB1, UB3, and UB4, during anaerobic switch-off. Crude extracts were prepared from cells before anaerobiosis (lanes 1), 10 min after anaerobiosis (lanes 2), and 10 min after cells were returned to a microaerobic condition (lanes 3).

genase reductase or to demodify it during the assay (data not shown).

Second, when the assay method does not allow reactivation during the assay itself, both strains UB1 (wild type) and UB4 (*draG*) display similar amounts of ADP-ribosylated dinitrogenase reductase following an anaerobic shift. TCA precipitation prevents reactivation by denaturing proteins immediately and allowing their quick extraction. As described in Materials and Methods, the use of SDS-PAGE allows the separation and quantitation of the modified and unmodified subunits of dinitrogenase reductase. While other implications of the data in Fig. 2 are discussed below, for this argument the critical data are shown in panel B, lanes 2, for both strains UB1 and UB4. The fact that similar proportions of modified dinitrogenase reductase were found in UB1 (wild type) compared with UB4 (*draG*) following anaerobic switch-off confirms that the higher residual nitrogenase activity in UB1 (Fig. 1) results from reactivation during that assay.

Because of the demodification of dinitrogenase reductase during the assay itself, very short assay times were employed in all subsequent experiments, except as noted.

The data in Fig. 2B also show that nitrogenase activity correlates with the degree of unmodified dinitrogenase reductase in the cell. For example, there is no modification of dinitrogenase reductase in UB1 (wild type) prior to the shift to anaerobic conditions, as evidenced by the absence of the modified subunit (lane 1). Ten minutes after the shift, the modified subunit of dinitrogenase reductase became apparent (lane 2), but it disappeared upon a return to microaerobic conditions (lane 3). As expected, strain UB3 (*draT*) showed no ability to modify dinitrogenase reductase, whereas strain UB4 (*draG*) modified the protein in response to the shift but was unable to reverse the modification. UB4 does show a small amount of modified dinitrogenase reductase before a shift to anaerobic conditions; this is consistent with an extremely low level of DRAT activity (and a complete lack of DRAG) under these conditions. These data show clearly that the DRAT-DRAG system is responsible for the bulk of the regulation of nitrogenase activity in response to anaerobic switch-off in *A. brasilense*.

NH_4^+ switch-off of nitrogenase. NH_4^+ switch-off of nitrogenase activity in UB1 (wild type), UB3 (*draT*), and UB4 (*draG*) has been studied previously (33), and the results were similar to those reported here for an anaerobic shift. UB1 (wild type) lost nitrogenase activity after the addition of NH_4^+ but recovered after NH_4^+ depletion. UB4 (*draG*) switched off nitrogenase activity but was unable to recover activity. UB3 (*draT*) showed no regulation by the added NH_4^+. We now confirm that those effects reflect ADP-ribosylation of dinitrogenase reductase.

In the experiment shown in Fig. 2A, the level of NH_4^+ supplied (250 μM) was sufficiently low that it was metabolized by the culture during the time of the experiment. As above, UB1 (wild type) exhibits the modified subunit of dinitrogenase reductase following NH_4^+ addition, and this modification is lost within 15 min, reflecting exhaustion of the NH_4^+. In UB3 (*draT*) no modification was found after NH_4^+ addition. Strain UB4 (*draG*) showed very little modified subunit prior to NH_4^+ addition; normal modification followed the addition of NH_4^+, but no demodification followed exhaustion of NH_4^+.

DRAT is transiently activated following an anaerobic shift. As shown previously strains UB1 (wild type) and UB4 (*draG*) showed complete loss of nitrogenase activity in response to the

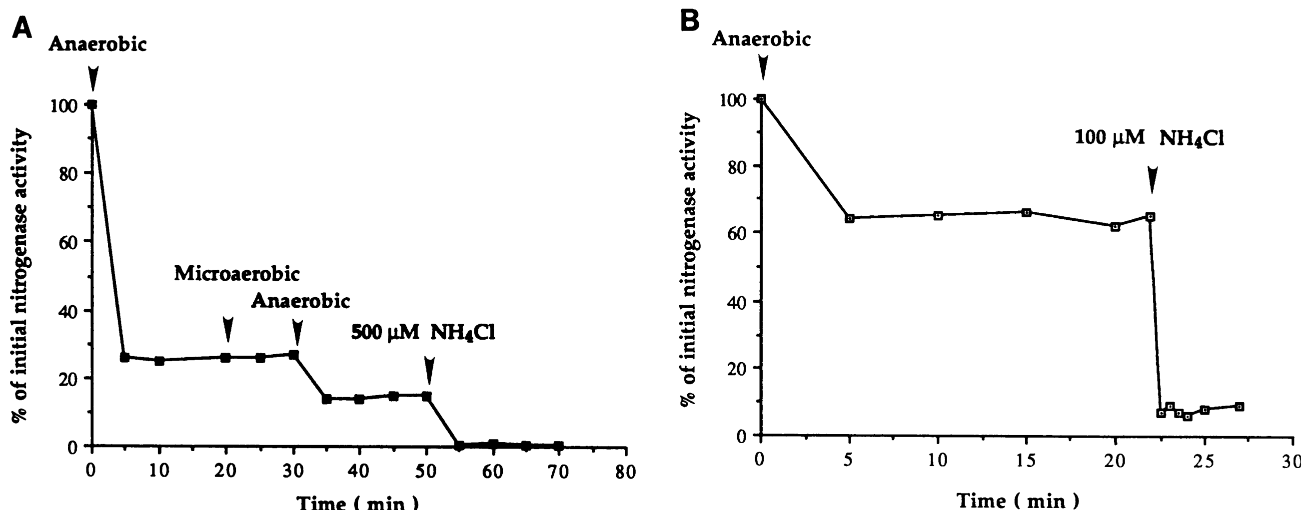

FIG. 3. Effects of successive rounds of switch-off conditions. (A) Effect of a combination of stimuli on nitrogenase activity in UB4 (*draG*). N_2-fixing UB4 cells were shifted to anaerobic conditions at time zero, and the cells were returned to microaerobic conditions at 20 min. After another 10 min, the cells were returned to an anaerobic condition. At 50 min, NH_4Cl was anaerobically added to a final concentration of 0.5 mM. A 1-ml sample of the cells was withdrawn and assayed for nitrogenase activity for 2 min, at the times indicated. (B) Effect of combination of stimuli on nitrogenase activity in UB1 (wild type). *nif*-derepressed cells were made anaerobic at time zero, and at 22 min NH_4Cl was added anaerobically to a final concentration of 100 μM. One-milliliter samples of the cells were withdrawn to assay nitrogenase activity for 0.5 min under a microaerobic condition at the times indicated.

addition of 0.5 mM NH_4^+ (33), in contrast to the partial loss of activity in response to anaerobiosis. We investigated this residual nitrogenase activity (following anaerobic shift) to determine how it might change with successive exposures to NH_4^+ or anaerobiosis.

Repeated exposures to these stimuli produced successive rounds of modification of dinitrogenase reductase. Strain UB4 (*draG*) was used to avoid reactivation during the nitrogenase assays, and it displayed little modified dinitrogenase reductase prior to stimulus (Fig. 2B). The culture displayed about 25% residual nitrogenase activity (Fig. 3A) after a shift to anaerobic conditions. After 20 min, the cells were incubated for 10 min under microaerobic conditions, during which there was no change in the residual nitrogenase activity. A second anaerobic treatment caused a further reduction in nitrogenase activity to about 12%. After 50 min, NH_4Cl was added anaerobically to a final concentration of 0.5 mM; this completely eliminated nitrogenase activity.

UB1 (wild type) gave similar results (Fig. 3B), although the presence of active DRAG in this strain produced higher residual nitrogenase activity, as seen before. This residual nitrogenase activity was almost abolished by 100 μM NH_4Cl. It was impossible to perform a meaningful second cycle of anaerobic treatment with UB1 (wild type) as its active DRAG would support complete recovery of nitrogenase activity during the intervening microaerobic period. The observation that UB1 responded independently to two different stimuli shows that this effect is not an artifact resulting from the absence of DRAG.

Quantitation of the modified and unmodified subunits of dinitrogenase reductase in both strains during the above experiments demonstrated incomplete modification after the first stimulus and further modification following the subsequent stimuli (data not shown).

These results are consistent with the following model: an anaerobic shift activates DRAT only transiently, and DRAT activity is lost before the ADP-ribosylation of dinitrogenase reductase is complete, yielding a residual level of nitrogenase activity. Successive rounds of negative stimuli apparently provide new periods of transient DRAT activation and therefore further modification of dinitrogenase reductase and a lower residual nitrogenase activity. Because DRAT loses its activity even while anaerobic conditions are maintained, the signal for its activation, or for some other factor necessary for modification of dinitrogenase reductase, must be depleted during this period. This indicates that the signal leading to DRAT activation is the shift to anaerobiosis, rather than the anaerobic state itself. An alternative model, which we cannot eliminate, is that all NAD, the required substrate for ADP-ribosylation, is reduced under anaerobic conditions, eliminating modification.

Response of DRAT activity to NH_4^+ addition. We recognized that the activation of DRAT in response to an anaerobic shift was transient because there was a residual level of nitrogenase activity after response to this stimulus. In contrast, our previous results showed that high levels of NH_4^+ led to a complete loss of nitrogenase activity (33). We examined the effect of lower levels of NH_4^+ to see whether this would provide an insight into the nature of DRAT activation. Figure 4A demonstrates that the addition of low levels of NH_4^+ (30 to 100 μM) to strain UB4 (*draG*) decreased residual nitrogenase activity to levels that correlated roughly with the amount of NH_4^+ added. This residual nitrogenase activity can be reduced further by the subsequent addition of a high concentration of NH_4^+ (data not shown). The degree of modification of dinitrogenase reductase in these experiments was monitored by Western immunoblot analysis; conversion of the dinitrogenase reductase pattern from one or two bands correlated with the nitrogenase activity observed. These results indicate that, in response to low levels of NH_4^+, DRAT loses activity before modification of dinitrogenase reductase is complete.

Although several models may explain this transient response to low levels of NH_4^+, we favor a model in which DRAT is substantially activated by the NH_4^+ signal but becomes inactive as the NH_4^+ is metabolized. Because high levels of NH_4^+

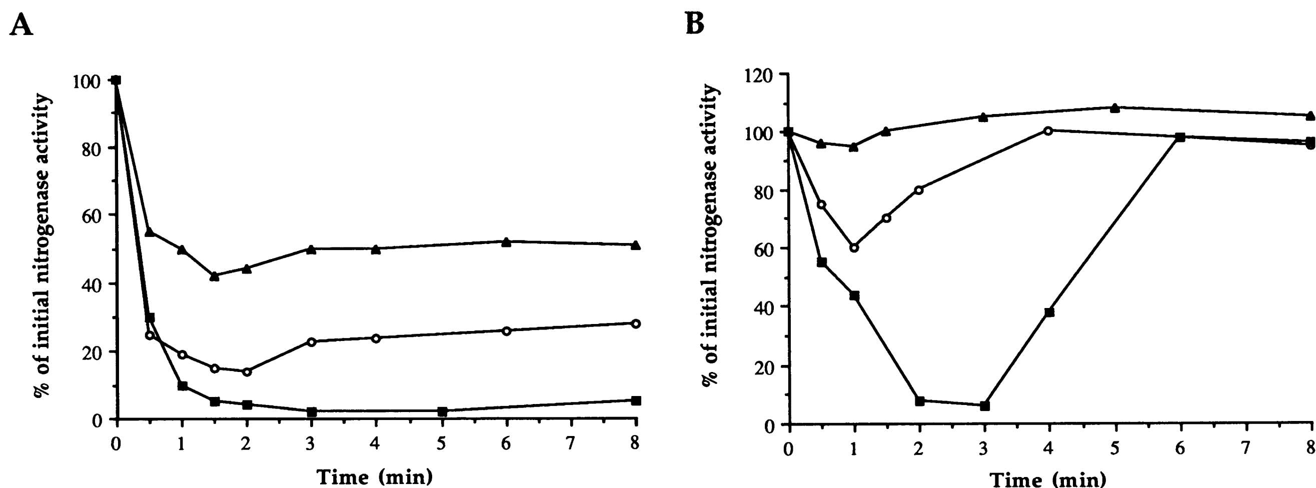

FIG. 4. Regulation of nitrogenase activity by low concentrations of NH_4Cl in strains UB4 (*draG*) (A) and UB1 (wild type) (B). At time zero, NH_4Cl was added to a final concentration of 30 μM (▲), 50 μM (○), or 100 μM (■). One-milliliter samples of the cells were withdrawn and assayed for nitrogenase for 0.5 min at the times indicated. There was no effect from adding water as a control (data not shown). Initial activity was the same as in Fig. 1.

led to a complete loss of nitrogenase activity, in contrast to anaerobiosis, a high level of NH_4^+ apparently sends a signal that is stronger or more-long-lived than that with an anaerobic shift. We have been unable to determine whether DRAT is permanently activated in response to high levels of NH_4^+, because there remains no unmodified dinitrogenase reductase as a monitor for DRAT activity in the cell.

It is interesting that in both *Rhodobacter capsulatus* (25) and in *R. rubrum* (14), high levels of NH_4^+ result in only partial ADP-ribosylation of dinitrogenase reductase, suggesting that DRAT is only transiently activated in response to this stimulus. This hypothesis is supported by the observation that the addition of a second negative stimulus resulted in a further round of modification.

DRAG is substantially active throughout switch-on conditions. Because of the surprising loss of DRAT activity after its transient activation, we also examined the timing of DRAG activation and inactivation. To determine whether DRAG is active in *nif*-derepressed cells (i.e., switch-on conditions) before switch-off, we examined the kinetics of switch-off in the presence or absence of DRAG. We reasoned that if DRAG were only transiently active, it should become inactive after a long period of switch-on conditions; following a negative stimulus, DRAT would be activated and should modify dinitrogenase reductase without any demodification by the inactive DRAG. However, if DRAG remains active under switch-on conditions, then a negative stimulus would lead to the activation of DRAT and inactivation of DRAG; the initial presence of active DRAG should reverse the effect of the newly active DRAT to some extent. In such a situation, therefore, a $DraG^-$ mutant should show more-complete or faster modification than wild-type cells, because there is no DRAG to compete with the newly activated DRAT. These possibilities were examined in the following three experiments.

In the first, an experiment similar to that for which the results are shown in Fig. 4A (with UB4, *draG*) was repeated with UB1 (wild type) (Fig. 4B). The strains used in the two experiments differ only with respect to the presence of DRAG; presumably their ability to take up and metabolize NH_4^+ and their regulation of DRAT were identical. Low levels of NH_4^+ had much less effect on nitrogenase activity in UB1 (wild type) (Fig. 4B) than in UB4 (*draG*) (Fig. 4A); addition of 30 μM NH_4^+ showed essentially no effect on UB1. This result supports the model that DRAG was active in UB1 at the time that NH_4^+ was added. Apparently, inactivation of DRAG began in response to the addition of NH_4^+, but NH_4^+ was depleted before a substantial fraction of the dinitrogenase reductase was modified.

The presence of active DRAG at the time that NH_4^+ was added should also cause a slower net modification of dinitrogenase reductase, because of the simultaneous reactivation of dinitrogenase reductase by DRAG. Figure 5 shows a comparison of the rates of response to NH_4^+ by UB1 (wild type) and UB4 (*draG*). Consistent with the model, UB4 (*draG*) showed faster loss of nitrogenase activity and modification of dinitrogenase reductase than did UB1 (wild type).

Finally, whereas UB4 (*draG*) shows a low accumulation of modified dinitrogenase reductase under switch-on conditions, UB1 (wild type) shows no such accumulation (Fig. 2). This suggests that there is a trace level of DRAT activity under these conditions but that it is completely compensated for by DRAG activity in UB1. The results of all these experiments indicate that DRAG is substantially active under switch-on conditions.

In *R. rubrum*, a comparison of a *draG* mutant with the wild type showed that the mutant responded with a faster loss of nitrogenase activity in response to a negative stimulus (13). Similarly, the *draG* mutant showed a low level of modified dinitrogenase reductase during switch-on conditions (13). This suggests that DRAG is substantially active under switch-on conditions in this organism also.

DRAG is inactive under steady-state anaerobic conditions. The pulse-chase experiment (8) noted in the Introduction showed that a high concentration of NH_4^+ added to *R. rubrum* effected complete, long-term elimination of DRAG activity. Figure 1 also indicates that DRAG is completely inactive in *A. brasilense* following anaerobic switch-off. Specifically, strains UB1 (wild type) and UB4 (*draG*) each show a stable residual activity of nitrogenase under anaerobic conditions. Because DRAT is only transiently active following anaerobic treatment, the stable level of nitrogenase activity that is eventually reached indicates that DRAG is also inactive at this time. Note

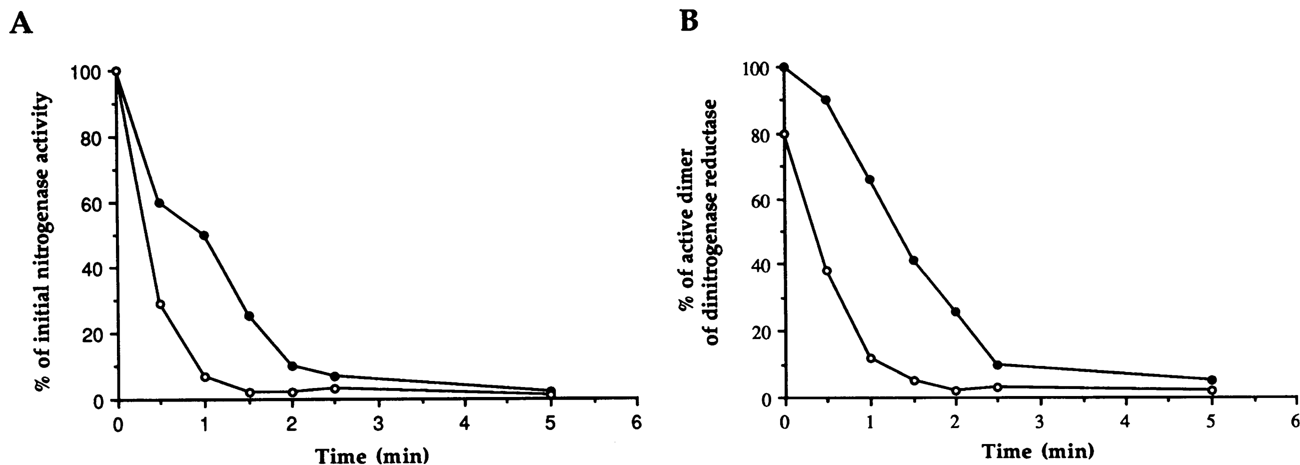

FIG. 5. Rates of response of *A. brasilense* strains to NH_4^+ switch-off. Rates of decrease (A) and modification (B) of nitrogenase activity during NH_4^+ switch-off in UB1 (wild type) (●) and UB4 (*draG*) (○). At time zero, NH_4Cl was added to a final concentration of 0.5 mM. At the times indicated, 1-ml samples of cells were withdrawn. For panel A, the samples were assayed for nitrogenase activity for 0.5 min. Initial activity was the same as in Fig. 1. For panel B, the samples were precipitated with TCA. After SDS-PAGE and immunoblotting, the protein bands were scanned and the percentage of active dimer of dinitrogenase reductase was calculated as described in Materials and Methods.

that DRAG activity does not return even when DRAT activity disappears due to exhaustion of the signal for its activation.

As a further confirmation that DRAG is inactive under anaerobic conditions, we compared the effect of NH_4^+ on anaerobically treated UB1 (wild type, Fig. 3B) to microaerobically grown UB1 and UB4 (*draG*, Fig. 5). The responses of anaerobically treated UB1 and microaerobically grown UB4 were similar, and both responses were somewhat faster than those of microaerobically grown UB1. Apparently, DRAG is inactive in anaerobically treated UB1 (wild type) but is always active during switch-on conditions.

What are the metabolic signals for DRAG and DRAT regulation? There have been several attempts to identify a single metabolite that could serve as a general signal for the DRAG-DRAT system. Since DRAT requires NAD for ADP-ribosylation, NAD was a potential regulatory metabolite, although the NAD and NADH pools in *R. rubrum* appear to be stable during NH_4^+ switch-off (21). While it was reported that exogenous NAD can inhibit nitrogenase activity partially in *R. rubrum* (28), we detected no significant inhibition in *A. brasilense* (data not shown). Because of their effects on DRAG and DRAT in vitro (16, 22), Mg^{2+} and Mn^{2+} were also regulatory candidates. Indeed, it has been reported that Mn^{2+} is an essential trace element for N_2 fixation and nitrogenase regulation in both *R. rubrum* and *R. capsulatus* (32). However, exogenous Mg^{2+} and Mn^{2+} failed to show any significant effects on regulation of nitrogenase activity in *A. brasilense* (data not shown). Finally, although Hartmann et al. (6) reported that the glutamine pool changed during NH_4^+ switch-off but not during anaerobic switch-off, the role of glutamine in NH_4^+ switch-off still is not established clearly in any nitrogen-fixing organism.

It is possible that energy may play a role in anaerobic switch-off. *A. brasilense* can use nitrate anaerobically as an alternative electron acceptor and to support nitrogenase activity (2, 20). We studied the effect of nitrate on nitrogenase activity in UB1 (wild type), UB3 (*draT*), and UB4 (*draG*), following a shift to anaerobic conditions, and the results are shown in Fig. 6. When $NaNO_3$ was added at a final concentration of 20 mM to *nif*-derepressed, anaerobically treated UB1 (wild-type) or UB3 (*draT*) cells, they were able to utilize NO_3^- to support nitrogenase activity after a 30-min lag. Strain UB3 showed slightly higher nitrogenase activity than did UB1, because UB3 is unable to modify dinitrogenase reductase in response to the anaerobiosis. In contrast, strain UB4 (*draG*) displayed only a small recovery of nitrogenase activity in the presence of NO_3^-, since it lacks DRAG and therefore cannot reactivate the ADP-ribosylated dinitrogenase reductase (Fig. 6A). The direct measure of ADP-ribosylation of dinitrogenase reductase on SDS-PAGE showed that demodification occurred when nitrogenase activity was recovered (data not shown). The loss of nitrogenase activity after 2 h is probably due to the accumulation of NO_2^-, which inhibits nitrogenase activity (2), although degradation of dinitrogenase reductase in these strains also was observed after 2 h of exposure to NO_3^- (detected by the SDS-PAGE analysis). Without the addition of NO_3^- following the shift to anaerobiosis, all three strains possessed very low, but stable, nitrogenase activity (Fig. 6B). These results suggest that the elaboration of alternate cell machinery for energy generation is both necessary and sufficient to support the reactivation of nitrogenase. Taken together with the results with NH_4^+, these results suggest that the signal for the DRAG-DRAT system may reflect a balance among multiple compounds, rather than the level of single compound in the cell.

Although we cannot identify the specific effector signals involved in reversible regulation in *A. brasilense*, our studies give some insight into the effects of those signals on DRAG and DRAT. When DRAG and DRAT appeared to be coordinately, though inversely regulated, it seemed possible that a particular signal caused the simultaneous activation of DRAT and inactivation of DRAG, whereas the absence of that signal caused the reverse. However, the present work has identified conditions under which DRAG and DRAT are not inversely regulated; specifically, under steady-state anaerobic conditions both DRAG and DRAT are inactive. This precludes the simple model and demands a more complicated signalling pathway for the two enzymes.

This report is the first to indicate the existence of a transient period when both DRAT and DRAG are active. This argument is based on two experiments: the difference in response to low levels of NH_4^+ (30 μM) between UB4 (*draG*) and UB1

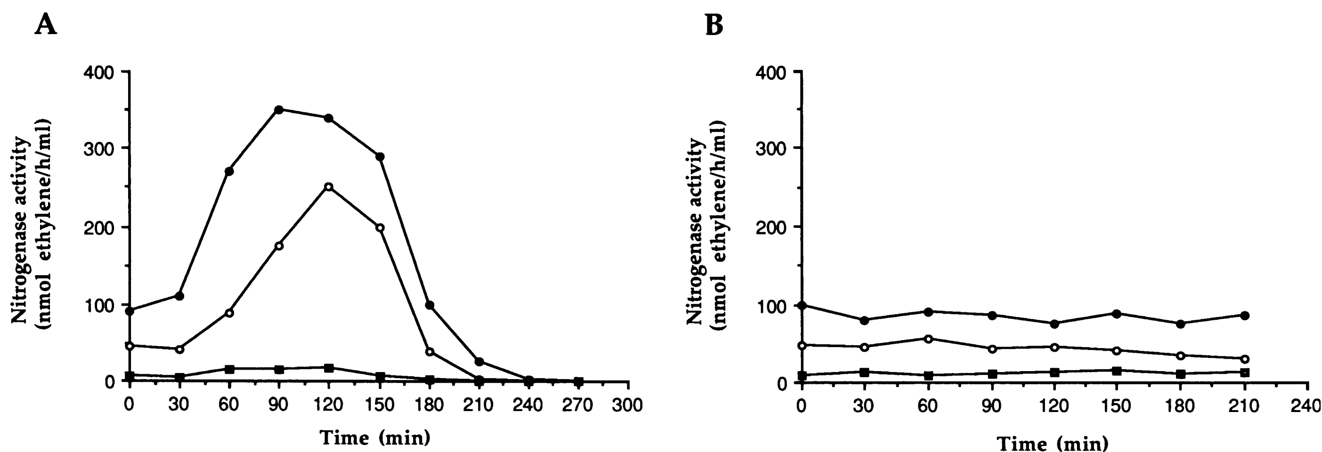

FIG. 6. Effect of NO_3^- on nitrogenase activity of anaerobically treated *A. brasilense* UB1 (wild type), UB3 (*draT*), and UB4 (*draG*) strains. Forty milliliters (each) of microaerobic nitrogen-fixing culture of UB1 (○), UB3 (●), and UB4 (■) was transferred to stoppered 120-ml bottles and made anaerobic as described in Materials and Methods. Anaerobic $NaNO_3$ solution was added to a final concentration of 20 mM at time zero (A), while water was added to the control (B). Samples (1 ml) were withdrawn anaerobically and assayed for nitrogenase activity under anaerobic conditions for 5 min (air in the assay vial was replaced with argon).

(wild type) (Fig. 4) and the difference in rates of loss of nitrogenase activity and modification of dinitrogenase reductase in these two strains (Fig. 5). In each case, the difference seen between the two strains following a negative stimulus indicates the presence of some DRAG activity (in wild type) after that point and therefore of a futile cycle of modification and demodification of dinitrogenase reductase. As dinitrogenase reductase is abundant in the cell (approximately 100 μM), such a cycle potentially can deplete the NAD pool in the cell if it operates for an extended period.

Site of DRAG-DRAT regulation. It is possible that the apparent regulation of DRAG and DRAT activities is effected through changes in their accessibility to their substrate, dinitrogenase reductase, rather than through direct modulation of their activity. This would explain how DRAG and DRAT may appear to be active in cell extracts, regardless of the conditions under which the cells were grown (15, 31). However, the sequential modifications indicated by the results shown in Fig. 3A appear to argue against this model. Our present information is inadequate to identify the site of DRAG-DRAT regulation unequivocally.

Conclusions. The experiments described establish a number of features of the switch-off response in *A. brasilense*. (i) Anaerobic and NH_4^+ regulation of nitrogenase depends on DRAG and DRAT, and the effects correlate with the ADP-ribosylation of dinitrogenase reductase. This does not rule out other regulatory mechanisms, as recently reported for NH_4^+ switch-off in *R. capsulatus* (25). (ii) DRAT is only transiently regulated in response to a shift to anaerobic conditions, whereas the duration DRAT activation in response to high levels of NH_4^+ cannot be determined easily in this organism. (iii) There are conditions (e.g., steady-state anaerobiosis) in which DRAG and DRAT activities are not inversely controlled. (iv) A futile cycle, when both DRAG and DRAT are active, appears to function for a short time following introduction of a switch-off stimulus.

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