JOURNAL OF BACTERIOLOGY, Mar. 1993, p. 1358–1366
0021-9193/93/051358-09$02.00/0

Vol. 175, No. 5

Posttranslational Regulation of Nitrogenase in *Rhodobacter capsulatus*: Existence of Two Independent Regulatory Effects of Ammonium

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Received 21 September 1992/Accepted 28 December 1992

In the photosynthetic bacterium *Rhodobacter capsulatus*, nitrogenase activity is regulated by ADP-ribosylation of component II in response to the addition of ammonium to cultures or to the removal of light. The ammonium stimulus results in a fast and almost complete inhibition of the in vivo acetylene reduction activity, termed switch-off, which is reversed after the ammonium is exhausted. In the present study of the response of cells to ammonium, ADP-ribosylation of component II occurred but could not account for the extent and timing of the inhibition of activity. The presence of an additional response was confirmed with strains expressing mutant component II proteins; although these proteins are not a substrate for ADP-ribosylation, the strains continued to exhibit a switch-off response to ammonium. This second regulatory response of nitrogenase to ammonium was found to be synchronous with ADP-ribosylation and was responsible for the bulk of the observed effects on nitrogenase activity. In comparison, ADP-ribosylation in *R. capsulatus* was found to be relatively slow and incomplete but responded independently to both known stimuli, darkness and ammonium. Based on the in vitro nitrogenase activity of both the wild type and strains whose component II proteins cannot be ADP-ribosylated, it seems likely that the second response blocks either the ATP or the electron supply to nitrogenase.

Nitrogenase is the enzymatic complex responsible for nitrogen fixation, a prokaryote-specific process that converts N_2 to NH_3. Two components constitute nitrogenase (see reference 4 for a review), component I (or dinitrogenase or MoFe-protein), which contains the active site for N_2 reduction; and component II (or dinitrogenase reductase or Fe-protein), whose main function in N_2 reduction is to pass electrons to component I, with the concomitant hydrolysis of MgATP. Since 20 to 30 molecules of MgATP are required for the fixation of 1 molecule of N_2 under normal conditions (4), it is not surprising that this very energy-demanding process is tightly regulated, sometimes at both the transcriptional and posttranslational levels. These regulatory effects are seen when fixed nitrogen becomes available in the medium or when energy becomes limiting. Transcriptional regulation occurs in every diazotroph studied and allows a response to fixed nitrogen or to oxygen through both global and *nif*-specific systems. It has been best characterized in *Klebsiella pneumoniae* (17), although it has been extensively studied in *Rhodobacter capsulatus* (11, 25, 27, 53). Posttranslational regulation of nitrogenase has also been found in various microorganisms (see reference 43 for a review) and results in the rapid response of nitrogenase activity to external stimuli.

The most extensively studied example of posttranslational regulation of nitrogenase is the ADP-ribosylation of component II in the photosynthetic bacterium *Rhodospirillum rubrum* (reviewed in references 43 and 57). In response to the addition of fixed nitrogen or to the removal of light, an ADP-ribose group is covalently attached to the arginine 101 residue of one of the component II subunits, causing inhibition of nitrogenase activity. This modification is performed by the enzyme dinitrogenase reductase ADP-ribosyl transferase (DRAT) (39). When the stimulus is exhausted or removed, the ADP-ribose moiety is removed from component II, restoring nitrogenase activity. This ADP-ribose removal is catalyzed by dinitrogenase reductase-activating glycohydrolase (DRAG) (59). For *R. rubrum* and *Azospirillum brasilense*, which is a microaerobic nitrogen-fixing bacterium, the *draT* and *draG* genes, coding for DRAT and DRAG, respectively have been cloned, sequenced, and expressed in different backgrounds (10, 12, 71). Mutations of these genes have been analyzed, and posttranslational regulation of nitrogenase in these mutants has been found to be strictly dependent on the presence of *draTG* (37, 71).

Posttranslational regulation of nitrogenase in the photosynthetic bacterium *Rhodobacter capsulatus* also involves ADP-ribosylation of component II (19, 29, 47). Removal of a culture from the light causes ADP-ribosylation (50), and addition of ammonium to cultures results in an immediate inhibition of nitrogenase activity (20, 23, 28, 68). However, none of these reports established a good correlation between ADP-ribosylation of component II and loss of activity resulting from the ammonium stimulus, either because the relative kinetics of the two effects were not determined or because the ADP-ribosylation was not quantified. Moreover, cells were often subjected to the other stimulus, darkness, during centrifugation steps.

We previously described mutants whose component II proteins are no longer substrates for ADP-ribosylation (50). The observation that their in vivo nitrogenase activity continued to respond to ammonium addition resulted in the detection of a second regulatory response that regulates nitrogenase activity in response to this stimulus.

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TABLE 1. *R. capsulatus* strains

Strain	Relevant genotype and comments[a]	Reference
B10	Wild type	44
RcM1(pJP2)	Δ*nifHDK*::*kan*; control strain carrying the plasmid-borne wild-type *nifHDK* operon; Km^r Tc^r	50
RcM1(pJP23)	Tyrosine mutant; Km^r Tc^r	50
RcM1(pJP24)	Phenylalanine mutant; Km^r Tc^r	50
RcM1(pJP27)	Leucine mutant; Km^r Tc^r	50

[a] The control strain which expresses a wild-type component II, in contrast to the tyrosine, phenylalanine, and leucine mutants, which carry substitutions replacing the arginine that is the normal target for ADP-ribosylation. These substitutions correspond to point mutations in *nifH*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study are listed in Table 1. Throughout the remainder of this article, we will refer to the various mutants with amino acid substitutions at Arg-102 by the specific amino acid replacement; thus, the strain with a tyrosine substitution will be termed the tyrosine mutant. As described previously (50), the tyrosine, phenylalanine, and leucine mutants must be compared with the control strain, RcM1(pJP2), because the location of the operon of the nitrogenase structural genes on the plasmid slightly modifies the sensitivity of the strains to the stimuli. Strains were routinely maintained on YPS plates (65) without antibiotics and grown in liquid RCV medium (65) containing 30 mM lactate and 15 mM $(NH_4)_2SO_4$ with antibiotics when required. Cells were derepressed for nitrogenase synthesis as described previously (50) in 20 or 50 ml of RCV medium containing glutamate (7 mM) as a poor nitrogen source. For nitrogenase posttranslational regulation studies, stimuli were applied in the following manner: light was removed by wrapping tubes or vials in aluminum foil, or ammonium was added as an anaerobic solution that was injected in volumes of less than 1% of the culture volume.

Estimation of relative amount of active component II. Samples (1 to 2 ml) were removed from the culture and collected aerobically by rapid centrifugation before being frozen as described previously (50). Throughout the handling, samples were maintained in the same illumination as the culture to prevent regulation in response to the dark until they were frozen in a dry ice-ethanol bath. Cell pellets were then treated aerobically as follows. Cells were broken by ultrasonic treatment in 200 μl of sonication buffer as described previously (50) and centrifuged for 2 min to remove the bulk of unbroken cells and membrane debris. Immunoprecipitation was performed as described by Kanemoto and Ludden (30) with the following modifications. Aerobic crude extracts from the previous step were incubated overnight at 4°C in microcentrifuge tubes with 30 μl of antibody against *R. rubrum* component II. The immunoprecipitates were collected by a 2-min centrifugation and washed in 100 μl of 100 mM MOPS (morpholinepropanesulfonic acid)–1 M NaCl (pH 7.5). Pellets were resuspended in 10 to 20 μl of fresh sodium dodecyl sulfate (SDS)-cocktail buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 2% β-mercaptoethanol, 3% SDS), heated at 95°C for 2 min, and centrifuged for 10 s. Supernatants were loaded on a 12% polyacrylamide–SDS gel (36), and proteins were visualized by Coomassie staining. Gels were equilibrated in water, and the positions of bands corresponding to the ADP-ribosylated subunits (also referred to as modified [M] subunits) and unmodified (U) subunits were identified by comparison with results from the immunoprecipitation of a crude extract from the wild type that had not been derepressed for nitrogenase synthesis. Immunoblotting of one lane also confirmed the antigenicity of these two bands, while the absence of antigenically related bands, which would have comigrated with component II bands on these gels, was verified by comparison with the pattern of the immunoprecipitation of a tyrosine mutant extract, whose component II exhibits a shift in apparent molecular weight (50). Component II protein bands were quantified by densitometer scanning as described by Kanemoto and Ludden (30), and the percentage of active dimer in each sample was calculated as described by Lowery et al. (40).

Nitrogenase activity assays. In vivo acetylene reduction activity was monitored by a 1-min assay in the light, as described previously (50). Throughout this procedure, as well as those described below, samples were maintained in the same illumination as the culture (light intensity of 100 W m^{-2} or in the dark) until the assay was performed at 30°C with an illumination of 100 to 150 W m^{-2}.

In vitro activities in permeabilized cells were assayed in the light for 20 min, as described previously (50), with the following modifications: 600 μl of culture, incubated in the light for 5 min with 10 μl of toluene and 0.5 mM $Na_2S_2O_4$, was added to 400 μl of an ATP-regenerating system-artificial electron donor mixture. This mixture (pH 7.8) consisted of 100 mM MOPS, 12.5 mM ATP, 80 mM creatine phosphate, 12.5 U of creatine phosphokinase, and 25 mM $Na_2S_2O_4$ and contained different $MgCl_2$ and $MnCl_2$ concentrations: 10 mM $MgCl_2$ for nonactivating conditions, in which DRAG is known to be inactive, and 25 mM $MgCl_2$ and 0.5 mM $MnCl_2$ for activating conditions, in which DRAG is capable of activating modified component II (41, 42).

Specific acetylene reduction activities are reported in nanomoles per minute per one-half unit of optical density at 660 nm (OD_{660}), which corresponds approximately to nanomoles per minute per milligram (dry weight) (45).

RESULTS

Existence of a second regulatory response of nitrogenase activity to ammonium chloride that does not involve ADP-ribosylation of component II. The decrease in the level of non-ADP-ribosylated component II dimers was compared with the loss of in vivo acetylene reduction activity in two independent experiments with wild-type *R. capsulatus* (Fig. 1). There was a decrease in both nitrogenase activity and the amount of non-ADP-ribosylated component II in response to darkness. These decreases followed a similar time course to reach slightly different, slowly decreasing plateaus after 20 min. As explained previously (50), ADP-ribosylated component II is partially reactivated by DRAG during the acetylene reduction activity assay itself, since this assay must be performed in the light to supply cells with the energy required for nitrogenase activity. The apparent nitrogenase activity is therefore artifactually high relative to that in the sample used for ADP-ribosylation status analysis, because the latter was frozen immediately, with no time for reactivation to occur; the constant discrepancy between the level of unmodified component II and residual activity may be an indication of this constant reactivation of component II that occurs in the light. Since it has previously been shown that the ADP-ribosylation of wild-type component II is responsible for the majority of the effects on activity occurring in response to darkness (50), the correlation between nitroge-

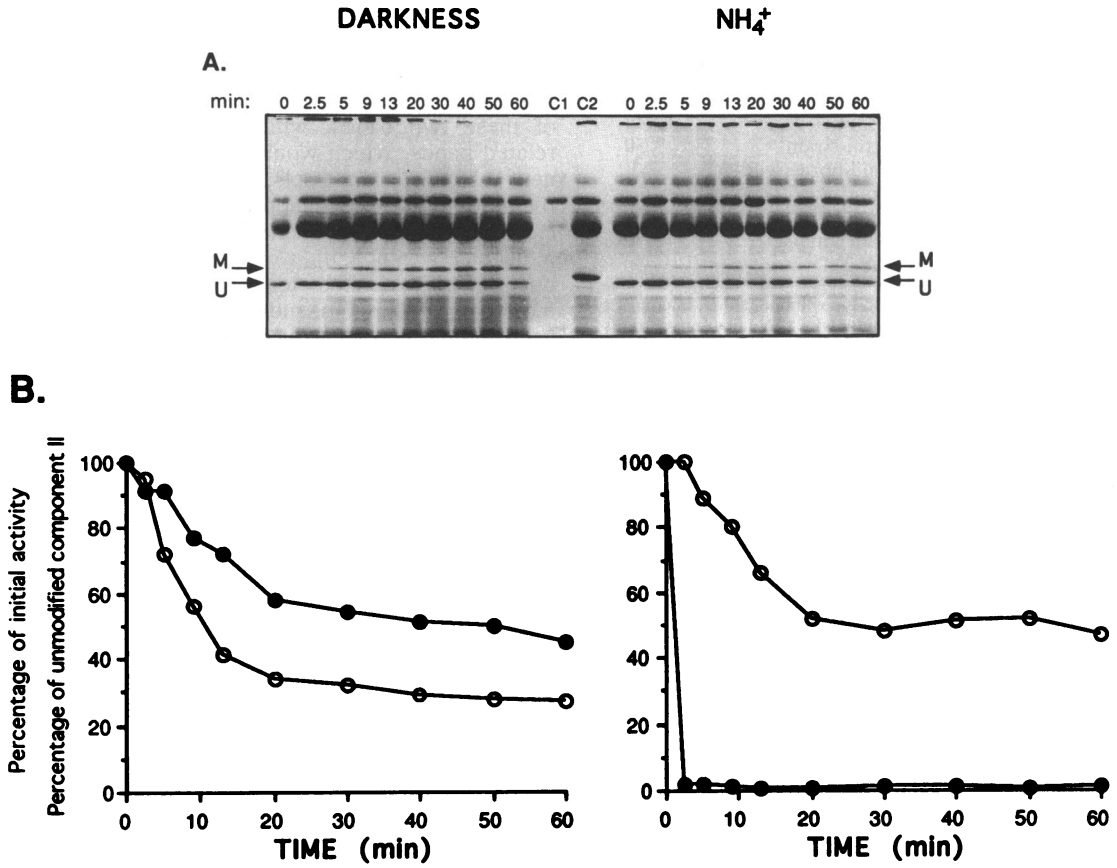

FIG. 1. Effect of darkness and 15 mM ammonium chloride on the ADP-ribosylation status of component II dimers and whole-cell acetylene reduction activity of the wild type. Both cultures were derepressed for nitrogenase synthesis to an OD_{660} of 1.6, corresponding to approximately 0.32 mg of protein per ml (45), and the stimulus (darkness or 15 mM ammonium chloride) was applied at time zero. For each time point, two 1-ml samples were quickly removed anaerobically; one sample was assayed for acetylene reduction activity for 1 min, and the other sample was treated by immunoprecipitation as described in Materials and Methods. (A) Coomassie-stained SDS-polyacrylamide gel, loaded with 10 μl of each sample, which consisted of immunoprecipitates solubilized in 15 μl of SDS-cocktail buffer. Lane C1 is a control with no cell extract added during the immunoprecipitation, and lane C2 is a sample processed with an extract prepared from a culture of the tyrosine mutant at an OD_{660} of 1.7, whose component II exhibits a shift in apparent molecular weight (50). The positions of the ADP-ribosylated (modified, M) and non-ADP-ribosylated (unmodified, U) subunits of wild-type component II are noted with arrows. (B) Plots of the residual acetylene reduction activity of cultures stimulated by darkness (left) and ammonium (right), expressed as a percentage of the initial activity (●), and the percentage of non-ADP-ribosylated component II dimers in the samples from each time point, calculated from the scan of the gel presented in panel A, as explained in Materials and Methods (○). Initial activities (100%) were 60 and 64 nmol min^{-1} $(OD_{660}/2)^{-1}$ in the cultures stimulated by darkness and by ammonium, respectively.

nase activity and level of non-ADP-ribosylated component II demonstrates that quantitation of the ADP-ribosylation status of component II is relatively precise.

A similar approach was used to analyze the effect of a 15 mM ammonium chloride stimulus (Fig. 1). Nitrogenase activity was almost completely eliminated in 2.5 min, whereas the ADP-ribosylation of component II resulted in modification of only 50% of the dimers within 30 min. The discrepancy between the residual level of activity and the level of unmodified component II and the great difference in the timing of the effects strongly suggest that the in vivo inhibition of acetylene reduction activity following ammonium chloride addition is not primarily the result of ADP-ribosylation.

To confirm the existence of a second response of nitrogenase activity to ammonium chloride, we studied the effect of this stimulus on *R. capsulatus* strains that possess nitrogenase activity but are no longer subject to ADP-ribosylation because the target arginine for this modification has been specifically altered by site-directed mutagenesis (50). Addition of anaerobic ammonium chloride to the cultures to a concentration of 300 μM resulted in a decrease in activity followed by a recovery upon ammonium exhaustion (Fig. 2). Both the extent and timing of this inhibition-recovery phenomenon depended somewhat on both the genotype and the culture density, but all the strains exhibited this profile, including the control strain expressing wild-type component II. Because the mutant component II proteins cannot be ADP-ribosylated, these results reflect a reversible effect on nitrogenase activity that is independent of ADP-ribosylation.

Second regulatory response involves ammonium as a stimulus. In order to demonstrate that the effect observed above resulted from ammonium ions, we examined the effects of other additions to cultures of the tyrosine mutant. Addition of either ammonium sulfate or ammonium acetate, at concentrations of 15 mM or 300 μM cation, had the same effect as addition of ammonium chloride, whereas addition of potassium chloride (15 mM or 300 μM) did not result in any

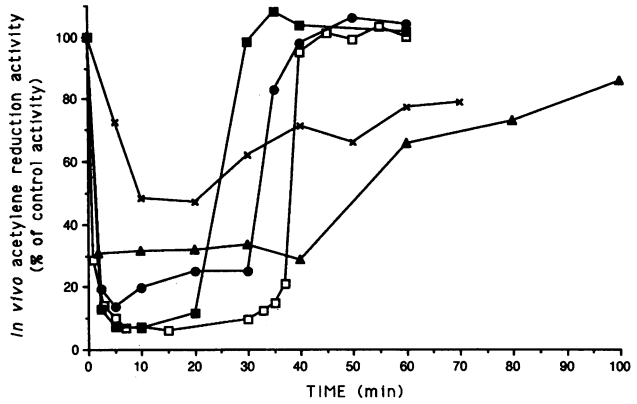

FIG. 2. Changes in the in vivo acetylene reduction activity of different cultures after addition of ammonium chloride or methylammonium chloride. Activities were assayed for 1 min except for the leucine mutant (3-min assay), and the results are reported as a percentage of the control activity, calculated by linear interpolation between two samples not exposed to any stimulus. The figure shows the effect of addition of 300 μM ammonium chloride on the control strain at an OD_{660} of 1.7 (□), the tyrosine mutant at an OD_{660} of 1.8 (■), the phenylalanine mutant at an OD_{660} of 1.75 (●), and the leucine mutant at an OD_{660} of 1.7 (▲) and the effect of addition of 300 μM methylammonium chloride on the tyrosine mutant at an OD_{660} of 1.8 (×). Initial activities were, respectively, 56, 32, 16, 5.2, and 41 nmol min^{-1} mg (dry weight)$^{-1}$.

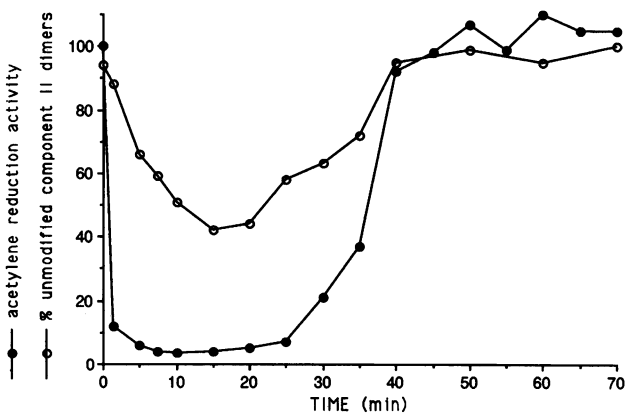

FIG. 3. Effect of 300 μM ammonium on the ADP-ribosylation status of component II and in vivo acetylene reduction activity of the wild type. A 50-ml culture was derepressed for nitrogenase synthesis in RCV-glutamate medium to an OD_{660} of 1.65, and a 1% culture volume of anaerobic 30 mM ammonium chloride solution was added at time zero. Sample analysis was carried out as described in the legend to Fig. 1. Residual acetylene reduction activity (●) is plotted as a percentage of the initial activity, which was 60 nmol/min/mg (dry weight), while the percentage of unmodified component II dimers in the samples from each time point (○) was calculated from the scan of an SDS gel of the immunoprecipitates (gel not shown).

significant change in the in vivo acetylene reduction activity (data not shown). The sensitivity of the second response was examined by adding very low ammonium concentrations to a culture of the tyrosine mutant (OD_{660} = 1.7). The results showed that 10 and 20 μM ammonium chloride did not produce any response, whereas 40 μM resulted in 83% inhibition of the activity after 2 min, followed by a complete recovery at between 5 and 10 min. Addition of 300 μM methylammonium to a culture of the tyrosine mutant also resulted in activity inhibition followed by recovery (Fig. 2), though the response was slower and less pronounced than with ammonium. Moreover, the recovery for the methylammonium effect was very slow; 50 min were necessary for recovery to 80% of the control activity. These results indicate that both ammonium and methylammonium can serve as stimuli for the second regulatory response of nitrogenase activity.

While ADP-ribosylation of component II was clearly not necessary for the observed effects on activity, we asked whether a low level (300 μM) of ammonium was capable of causing ADP-ribosylation of component II in the wild type. Figure 3 shows that this stimulus did trigger both the ADP-ribosylation of component II and a dramatic loss of nitrogenase activity; furthermore, both responses seemed to begin and end at approximately the same time. This synchronicity demonstrates that, although ADP-ribosylation is sensitive to this relatively low ammonium concentration, its effect is masked by the second response. Furthermore, this similarity suggests that both responses may use the same signal transduction pathway in response to ammonium.

The assimilation of ammonium through glutamine synthetase has been repeatedly suggested to be required for the triggering of ADP-ribosylation in several microorganisms (5, 8, 12, 14, 21, 26, 47, 66, 68, 70), although this has never been clearly demonstrated and is not supported by the results of Kanemoto and Ludden (31). We examined the response of the tyrosine mutant for insights into the possible role of glutamine in the signal transduction pathway. Unfortunately, the results were not conclusive because pretreatment of the culture with 0.5 to 3 mM DL-methionine sulfoximine, a glutamine synthetase inhibitor (46), inhibited nitrogenase activity by itself, without preventing the response to 300 μM ammonium. Moreover, glutamine appeared to prevent this inhibition by DL-methionine sulfoximine, probably by competing with the same transport systems.

These results demonstrate that ammonium and methylammonium are stimuli for a second response of nitrogenase activity. This response is sensitive to very small amounts of ammonium and is synchronized with ADP-ribosylation, although it is more efficient in terms of speed and extent of activity inhibition. Because of the experimental difficulty in discriminating between ammonium transport and its assimilation by glutamine synthetase (60), we are unable to identify the specific signal that causes this second response to ammonium.

Both responses of nitrogenase activity to ammonium or darkness are independent. As described above, ADP-ribosylation of component II is the only regulatory response triggered by darkness, whereas ammonium is the stimulus for two distinct responses. We therefore studied the effect of the combination of both stimuli on the two responses to test whether the stimulus effects were independent.

A wild-type culture was exposed first to 15 mM ammonium and then to darkness after 30 min, and both the nitrogenase activity and ADP-ribosylation status of component II were monitored (Fig. 4). Darkness caused ADP-ribosylation of an additional fraction of component II, indicating that the two stimuli were cumulative in terms of ADP-ribosylation. This further ADP-ribosylation was not a result of preferential degradation of unmodified component II, since reillumination of the culture caused the rapid reactivation of component II up to the level allowed by the

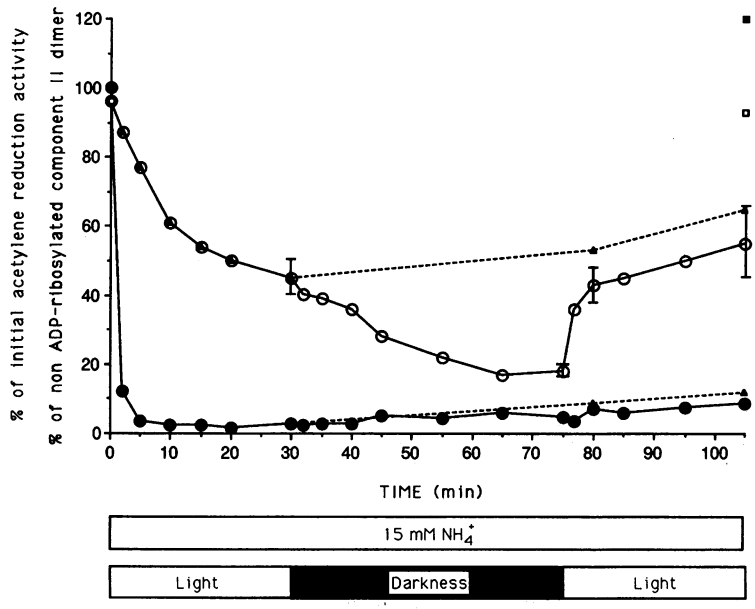

FIG. 4. Effect of a combination of stimuli on the ADP-ribosylation status of component II and the in vivo acetylene reduction activity of the wild type. A 55-ml culture was derepressed for nitrogenase synthesis in RCV-glutamate to an OD_{660} of 1.3, and 0.015 culture volume of 1 mM ammonium chloride was added at time zero. At 30 min, the cells were removed from the light for 45 min and then reilluminated at 75 min. Acetylene reduction activity was monitored by 1-min assays performed in the light, with samples removed from either the illuminated culture (time points 0 to 30 min and 77 to 110 min) or the nonilluminated culture (time points 32 to 75 min), and is reported as a percentage of the initial activity, which was 60 to 63 nmol min^{-1} mg (dry weight)$^{-1}$. ADP-ribosylation was monitored by immunoprecipitation and scanning of the SDS gel of the solubilized immunoprecipitates as described in Materials and Methods. Results from two independent experiments, one with fewer time points tested, are shown, and bars indicate the standard error observed for the points common to the two experiments: percentage of non-ADP-ribosylated component II dimers (○) and acetylene reduction activity (●). The responses of cultures exposed to ammonium only (▲, △) or to no stimulus (■, □) are also shown, with solid symbols representing acetylene reduction activity and open symbols representing the percentage of non-ADP-ribosylated component II.

ammonium stimulus alone. This last result also strongly suggests that the two stimuli for ADP-ribosylation do not use the same signal transduction pathway, since reillumination does not allow the level of non-ADP-ribosylated component II to recover beyond that in the ammonium-only control. The cause of the slow increase in the level of unmodified component II dimers in the control that remained illuminated is unknown.

The very low residual activity reflects the fraction of nitrogenase that remains unaffected by the action of either system. This activity also remained unaffected by darkness when quantified by a 1-min assay in the light, which is consistent with the fact that darkness is not a stimulus for the second response. A similar experiment in which the stimulus sequence was reversed (darkness-ammonium-light) was not conclusive because darkness appeared to prevent the active transport of ammonium, leaving only 1 min of light during the assay itself for the ammonium to be actively taken up and to induce the second response (data not shown).

Hypothesis concerning the mechanism involved in the second response to ammonium. We tested whether the second response involved a modification of nitrogenase by assaying nitrogenase activity in toluene-permeabilized cells that had been treated with ammonium. A comparison of these in vivo and in vitro activities for the wild type, control strain, and tyrosine and phenylalanine mutants in response to the ammonium stimulus is shown in Fig. 5.

Exposure of cultures to ammonium resulted in an inhibition of the in vivo nitrogenase specific activity, whose residual level was approximately the same for all strains, independent of their initial activity (Fig. 5A). The narrow range of values for these in vivo residual acetylene reduction activities was surprising and suggested that the rate-limiting step for nitrogenase activity had become the same in all these strains.

Figure 5B shows that the in vitro activities of the wild-type and control strains were 65% inhibited by ammonium, as determined under nonactivating conditions (defined in Materials and Methods). Under activating assay conditions, which did not affect the activity significantly when cells had not been exposed to ammonium, the inhibition resulting from exposure of the culture to ammonium could be substantially eliminated in vitro by adding Mn^{2+} and Mg^{2+} in concentrations known to be required for DRAG activity in an in vitro assay with purified proteins (41, 42, 51, 59). These results are consistent with the in vivo ADP-ribosylation of wild-type component II in response to ammonium and the in vitro reactivation of this protein by DRAG. They indicate that, despite the perturbations of cell handling and permeabilization, ADP-ribosylation apparently can account for all the inhibition of activity detected in vitro under these conditions; the effects of the second regulatory response are not detectable in the wild type or mutants under these conditions.

The failure to detect any in vitro effect of ammonium caused by the second regulatory response is consistent with the idea that this second response to ammonium does not involve a covalent modification of either of the nitrogenase components. It is also possible, however, that such a covalent modification might be lost upon permeabilization. Be-

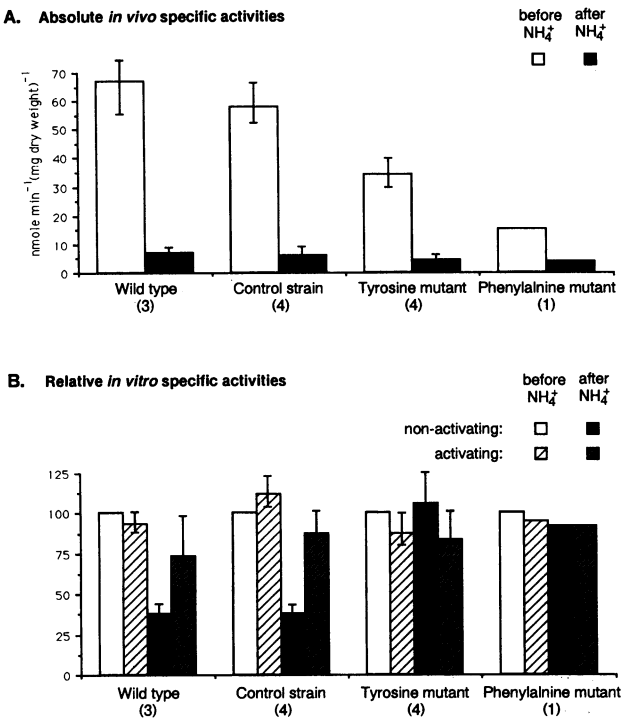

FIG. 5. Comparison of in vivo and in vitro specific acetylene reduction activities of the wild type, control strain, and tyrosine and phenylalanine mutants in response to 15 mM ammonium. Cultures (20 ml) were derepressed for nitrogenase synthesis in RCV-glutamate medium to an OD_{660} of between 1.2 and 2.1, and all sampling and handling procedures were performed with the same illumination as given the culture to prevent regulation in response to variation in light conditions. For each culture, two 8-ml samples were removed anaerobically, one before and one 20 min after anaerobic ammonium chloride was injected (15 mM final concentration), for determination of in vitro and in vivo activities. Both samples were then treated the same way and at the same time; while a 2-ml sample was later used for the whole-cell nitrogenase assay, a 6-ml sample was treated for permeabilization for 5 min, and 600-μl samples of permeabilized cells were distributed in assay vials containing the ATP-regenerating dithionite mixture. In vitro activities were determined in a 20-min assay performed after a 5-min preincubation, and in vivo activities were determined on intact whole cells simultaneously. Each value was the average of duplicates. (A) Absolute in vivo specific activities; (B) relative in vitro specific activities, normalized to the activity assayed in nonactivating conditions, with the sample taken before exposure to ammonium. Activating and nonactivating refer to assay conditions that stimulate or inhibit, respectively, removal of ADP-ribose from component II, as described in Materials and Methods. The absolute values for the no-ammonium, nonactivated activities were 3.5 nmol/min/mg for the wild type, 4.5 nmol/min/mg for the control strain, 9.1 nmol/min/mg for the tyrosine mutant, and 4.7 nmol/min/mg for the phenylalanine mutant. Each value represents an average for the number of experiments shown in parentheses. Bars indicate the range of values.

cause similar results with the tyrosine mutant were also obtained after cell breakage by sonication, any hypothesized loss of modification would not be specific to the permeabilization protocol.

DISCUSSION

In some diazotrophs, ADP-ribosylation of nitrogenase component II is a mechanism for the posttranslational regulation of nitrogenase in response to the addition of ammonium to the culture. In some phototrophs, this mechanism is also responsible for the reduction of nitrogenase activity seen when cultures are shifted from light to dark conditions. This work demonstrates the presence of another response of nitrogenase activity to ammonium addition in cultures of *R. capsulatus*. This second response is evident in the wild type, in which ADP-ribosylation of arginine 102 of component II cannot account for the timing and extent of the activity inhibition. The presence of this second response is also apparent in the ability of mutants whose altered components II are no longer substrates for ADP-ribosylation to respond to ammonium. In *R. capsulatus* wild type, in fact, this second response is responsible for the bulk of the observed effects on nitrogenase activity. Hence, the term "switch-off/switch-on" used by Zumft and Castillo (73), which refers only to changes in activity, must be used carefully, since these changes may result from more than one mechanism in a given organism.

The stimulus for this second response seems to be intracellular ammonium or its analog, methylammonium, but not darkness. Experiments that were performed to test the role of glutamine as a signal for the second response were inconclusive, mainly because uptake of ammonium is tightly coupled with its assimilation by the glutamine synthetase in *R. capsulatus* (60). The lower efficacy of the methylammonium transport system, which is present in these growth conditions, than of the ammonium transport system (54) could account for the timing of the response to methylammonium. However, the slower recovery from methylammonium than from ammonium cannot be taken as evidence for a role of glutamine or γ-glutamylmethylamide, the product of methylamine assimilation by glutamine synthetase (54, 68), in the signal transduction pathway, since the metabolic pathways for ammonium assimilation in *R. capsulatus* are not well understood (3).

It has recently been suggested that the *glnB* gene product, which is known to be important in responding to nitrogen status, might be involved in the signal transduction pathway during the switch-off following ammonium stimulus; the response of the in vivo nitrogenase activity was slower in mutants affected in the *glnB* gene than in the wild type (19). While the component II in these mutants accumulated in an ADP-ribosylated form, a good correlation between ADP-ribosylation and loss of activity was not demonstrated by these experiments because the relative kinetics of the two effects were not determined and because cells were exposed to the cumulative darkness stimulus during centrifugation. Given these concerns, we believe that it is premature to make firm conclusions about the role of the *glnB* product in either response to ammonium.

The molecular basis of this second response of nitrogenase activity to ammonium is unknown. As already suggested for other phototrophs (18, 73), inhibition of in vivo nitrogenase activity can result from ADP-ribosylation of component II, a depletion of the ATP pool resulting from a decrease in the membrane potential, or a decrease in the reductant supply to component II. The results of our in vitro assays of nitrogenase activity, with both the wild type and mutants that are not ADP-ribosylated, have demonstrated that the second response to ammonium does not result in inhibition of the in vitro nitrogenase activity, suggesting that the mechanism of the second response does not consist of any covalent modification of either of the nitrogenase components. As our in vitro assay provides both the ATP and reductant to component II, we cannot discriminate between the two remaining

hypotheses. However, results with *Rhodobacter sphaeroides*, a member of the family *Rhodospirillaceae* that is closely related to *R. capsulatus* (69), indicated that neither membrane potential nor ATP pool was affected by the addition of ammonium to the culture (18). This leaves reductant supply limitation as a candidate mechanism for the second response.

Such a hypothesis implies that the electron flow to component II is turned off in response to ammonium addition. This would likely occur by inactivation of an electron donor in the pathway. Our attempts to bypass this unknown electron donation step have been unsuccessful: adding 20 mM malate or pyruvate to a culture of the tyrosine mutant after exposure to 15 mM ammonium and assaying the in vivo acetylene reduction activity with argon-saturated vials containing 12% hydrogen did not change the level of residual activity (data not shown). It is possible that such inactivation might result from DRAT activity, although there is no evidence for this. A residual electron flux resulting from a pool of unmodified electron donors would constitute the rate-limiting step for nitrogenase activity during the observed in vivo inhibition phase. This would be consistent with the in vivo nitrogenase activities seen in the mutants and the wild type in response to ammonium.

It is interesting that, although the timing and extent of ADP-ribosylation in *R. capsulatus* is similar to that in *Rhodospirillum rubrum*, albeit with a higher residual level of unmodified component II (37), the effect on activity is more similar to that seen in *A. brasilense* (71). This might suggest the presence of a second response to ammonium in *A. brasilense* too, although ADP-ribosylation of nitrogenase component II in this bacterium is also very rapid (70).

The switch-off of nitrogenase activity in response to ammonium addition has been reported for many diazotrophs other than *Rhodospirillum rubrum*, *A. brasilense*, and *R. capsulatus* (1, 2, 5–7, 9, 13, 14, 16, 21, 24, 49, 52, 55, 56, 58, 61, 63, 72–74, and references below). However, in many of these reports, ADP-ribosylation of component II was not the apparent cause of the switch-off (14, 15, 18, 21, 22, 32–35, 38, 48, 62, 67). As a consequence, it could well be that post-translational regulation of nitrogenase activity occurs mainly via other mechanisms that remain to be elucidated.

To our knowledge, this is the first time that the existence of two synchronized responses of nitrogenase activity to ammonium has been reported to occur in the same bacterium. ADP-ribosylation of nitrogenase component II in *R. capsulatus* in response to ammonium appears to be redundant, since the second response is sensitive (down to 40 μM), faster, and more efficient in terms of effect on in vivo activity. Moreover, it is unclear why ADP-ribosylation responds independently to ammonium and darkness when combined, since the second response is the most efficient one when ammonium is present. However, it would appear that the primary utility of ADP-ribosylation in this organism is for response to light conditions, for which it appears to be the only regulatory response. Furthermore, it is also possible that other physiological conditions exist in which one or another of these mechanisms is the only one responsible for regulation of nitrogenase activity.

ACKNOWLEDGMENTS

J. Pierrard thanks C. Gaillardin for encouragement and support. We thank Y. Zhang, D. P. Lies, G. M. Nielsen, and R. L. Kerby for enlightening and fruitful discussions, J. L. Gaspar for technical assistance with some switch-off/on experiments, and also J. D. Wall for the gift of strain G29.

The collaboration of J. Pierrard with UW-Madison was undertaken during his National Service for Cooperation, sponsored by the French Atomic Energy Commission (CEA). The work was also supported by the College of Agricultural and Life Sciences, UW-Madison, and by grants 91-37305-6664 from the U.S. Department of Agriculture and MCB-8821820 from the NSF.

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