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Posttranslational Regulation of Nitrogenase in *Rhodospirillum rubrum* Strains Overexpressing the Regulatory Enzymes Dinitrogenase Reductase ADP-Ribosyltransferase and Dinitrogenase Reductase Activating Glycohydrolase

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***Rhodospirillum rubrum* strains that overexpress the enzymes involved in posttranslational nitrogenase regulation, dinitrogenase reductase ADP-ribosyltransferase (DRAT) and dinitrogenase reductase activating glycohydrolase (DRAG), were constructed, and the effect of this overexpression on in vivo DRAT and DRAG regulation was investigated. Broad-host-range plasmid constructs containing a fusion of the *R. rubrum nifH* promoter and translation initiation sequences to the second codon of *draT*, the first gene of the *dra* operon, were constructed. Overexpression plasmid constructs which overexpressed (i) only functional DRAT, (ii) only functional DRAG and presumably the putative downstream open reading frame (ORF)-encoded protein, or (iii) all three proteins were generated and introduced into wild-type *R. rubrum*. Overexpression of DRAT still allowed proper regulation of nitrogenase activity, with ADP-ribosylation of dinitrogenase reductase by DRAT occurring only upon dark or ammonium stimuli, suggesting that DRAT is still regulated upon overexpression. However, overexpression of DRAG and the downstream ORF altered nitrogenase regulation such that dinitrogenase reductase did not accumulate in the ADP-ribosylated form under inactivation conditions, suggesting that DRAG was constitutively active and that therefore DRAG regulation is altered upon overexpression. Proper DRAG regulation was observed in a strain overexpressing DRAT, DRAG, and the downstream ORF, suggesting that a proper balance of DRAT and DRAG levels is required for proper DRAG regulation.**

Nitrogen-fixing bacteria contain the nitrogenase enzyme complex, which catalyzes the reduction of atmospheric nitrogen to ammonium. The nitrogenase complex consists of two enzymes, dinitrogenase, an $\alpha_2\beta_2$ tetramer of the *nifK* and *nifD* gene products, and dinitrogenase reductase, an α_2 dimer of the *nifH* gene product. Dinitrogenase reductase transfers electrons, in an ATP-dependent manner, to dinitrogenase, which contains the site of substrate reduction. This process is very energy demanding and thus is controlled at the transcriptional level and in some systems also at the posttranslational level. Transcriptional control of *nif* gene expression is found in all nitrogen-fixing bacteria studied and is best characterized in *Klebsiella pneumoniae* (24). Posttranslational control of nitrogenase activity has been detected in a number of nitrogen-fixing bacteria, including *Rhodospirillum rubrum* (10, 23) and *Rhodobacter capsulatus* (14, 31) (both purple nonsulfur photosynthetic bacteria), *Chromatium vinosum* (a purple sulfur bacterium) (11), and *Azospirillum brasilense* (a microaerobic bacterium) (7, 12, 37, 38).

The nitrogenase posttranslational modification system is best characterized in *R. rubrum*. In response to environmental conditions such as darkness or introduction of a fixed nitrogen source like ammonium, an ADP-ribose group from NAD is covalently attached to arginine 101 on one subunit of the dinitrogenase reductase dimer (15). This modification disrupts electron transfer between dinitrogenase reductase and dinitrogenase and therefore inactivates nitrogenase-dependent substrate reduction (27). Dinitrogenase reductase ADP-ribosyltransferase (DRAT) catalyzes this ADP-ribosylation reaction (22). Nitrogenase activity is restored when the ADP-ribose group is cleaved by dinitrogenase reductase activating glycohydrolase (DRAG) upon exposure of cells to the energy source, light, or when the fixed-nitrogen source is exhausted (15, 33). Although it is known that the environmental signals of light, darkness, and fixed nitrogen are mediated through the DRAT-DRAG system, it is not known how these regulatory enzymes detect stimuli (8).

The following experiments have shown that DRAG and DRAT activities are themselves regulated posttranslationally in the cell. Regulation of DRAG was demonstrated in a pulse-chase experiment in which the turnover of the ^{32}P-labeled ADP-ribose group was monitored in *R. rubrum* cells treated with ammonium. In this experiment, [^{32}P]ADP-ribose turned over much more slowly than did the total phosphate pool. Because DRAG was present in the cells under these conditions, this experiment clearly showed that it was inactive (15). Regulation of DRAT was demonstrated by using a *draG* mutant of *R. rubrum*. In this strain, under nitrogenase-derepressing conditions, dinitrogenase reductase was active and not modified. Although DRAT was present, dinitrogenase reductase remained active and unmodified until the cells were exposed to darkness or ammonium (21).

Unfortunately for analysis of this nitrogenase regulation system, both DRAT and DRAG activities are always observed in cell extracts, regardless of the treatment of cells with light, darkness, or ammonium before extraction. Because the regulation of DRAT and DRAG is lost upon cell breakage, the role(s) of small molecules in DRAT and DRAG regulation has been investigated. The pools of adenylates, pyridine nucleo-

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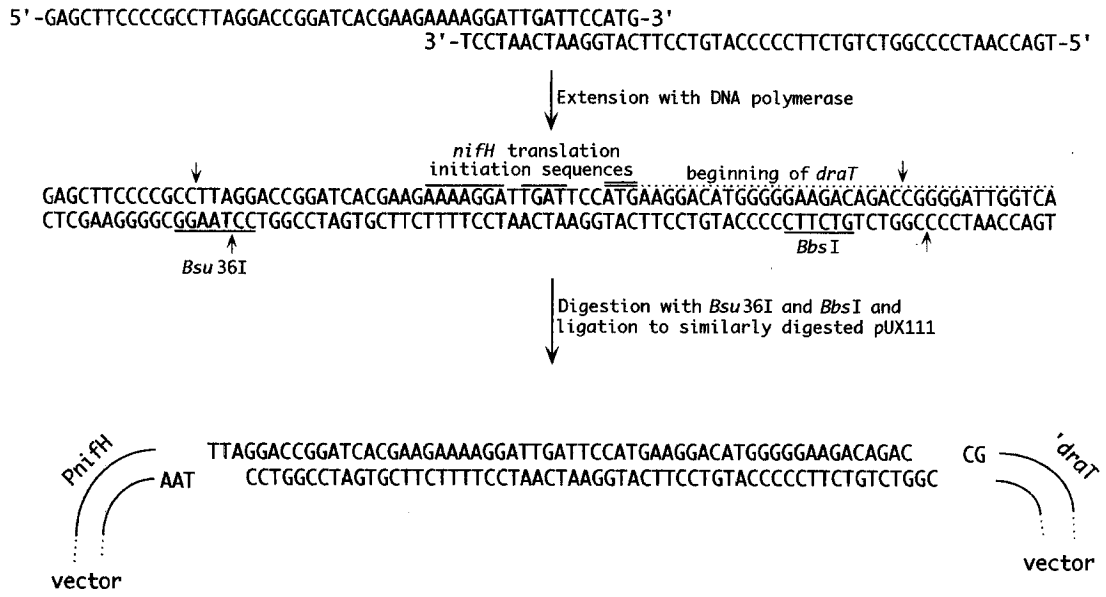

FIG. 1. Fusion of the *R. rubrum nifH* promoter region to the *draT* gene in pUX111. The double-stranded DNA fragment resulting from extension of the annealed oligonucleotides consists of bp 313 to 363 of the *nifH* region DNA sequence (19) and bp 46 to 80 of the *draT* DNA sequence (6). The translation initiation sequences have solid overlines in this DNA fragment, with single overlines indicating putative ribosome-binding sequences based on complementarity with the 3′ end of the *R. rubrum* 16S rRNA (9) and double overlines identifying the *nifH* methionine initiation codon. The *draT* coding region present on this fragment is indicated by a dotted overline. Recognition sequences for the restriction enzymes used to clone this fragment between the *nifH* promoter (P*nifH*) and the remainder of the *draT* gene (*draT*) are underlined, with the actual sites for enzyme cleavage of the DNA indicated by arrows. Details of the construction of this fusion are presented in Materials and Methods.

tides, metal ions, and amino acids have been analyzed, but no definite correlation has been found with nitrogenase regulation (15, 16, 20, 29, 30).

Mono-ADP-ribosylation is not unique to the nitrogen fixation system. It was first shown that diphtheria toxin ADP-ribosylates and thus inactivates the eukaryotic elongation factor 2 that is required for protein synthesis (13). Furthermore, the modes of action of cholera and pertussis toxins involve ADP-ribosylation of specific eukaryotic G proteins (26).

The *R. rubrum* genes encoding DRAT (*draT*) and DRAG (*draG*) have been cloned and sequenced (6). They are adjacent to each other and presumably are cotranscribed. Mutant analyses have demonstrated that *draT* and *draG* are responsible for nitrogenase posttranslational regulation and that an open reading frame (ORF) adjacent to and downstream of *draG* may also be involved in nitrogenase regulation (21).

Overexpression of the DRAT and DRAG proteins in *R. rubrum* was previously attempted by placing a restriction fragment containing the *nifH* promoter and the beginning of the *nifH* coding region from this organism upstream of the *dra* operon on a broad-host-range plasmid (28). The *R. rubrum* strain containing this plasmid construct overexpressed DRAG under *nif*-derepressing conditions but failed to overexpress DRAT, presumably because of interference with translation of the *draT* gene by translation of the *nifH* coding region placed upstream of *draT*. Recently, Durner et al. reported on the overexpression of functional DRAT and DRAG in *Escherichia coli* (4).

In this paper, we report on the construction of *R. rubrum* strains that overexpress the nitrogenase regulatory proteins, DRAT and DRAG. Through biochemical characterization of these strains, we studied how overexpression of these proteins affected nitrogenase regulation and therefore the regulation of DRAT and DRAG.

MATERIALS AND METHODS

Construction of overexpression plasmids. A DNA fragment encoding a perfect fusion of the *R. rubrum nifH* translation initiation sequences, including the *nifH* ATG translational start codon, to the second codon of *draT* was generated and used to place the *R. rubrum dra* genes under control of the *nifH* promoter (Fig. 1). Two 50-mer oligonucleotides with complementary 15-nucleotide sequences at their 3′ ends were designed such that extension from this overlap by DNA polymerase would yield a double-stranded DNA fragment containing the desired fusion and having restriction sites near its ends useful for linking the *nifH* promoter and *draT* gene.

The annealing and extension reactions were performed with Sequenase version 2.0 T7 DNA polymerase (United States Biochemical Corp., Cleveland, Ohio) in a modification of the protocols recommended by the manufacturer. The annealing reaction was performed as suggested, except that the only DNA added was 20 pmol of each oligonucleotide being used. In the extension reaction, all four nucleotide triphosphates were added to the reaction mixture at a final concentration of approximately 325 μM. The reaction was performed at 37°C for 10 min and terminated by heating for 10 min at 65°C. The extension reaction mixture was diluted approximately 6.5-fold with water, and a portion was digested simultaneously with the restriction enzymes *Bbs*I (New England Biolabs, Inc., Beverly, Mass.) and *Bsu36*I (Promega Corp., Madison, Wis.). A portion of this digested material was ligated to the purified large restriction fragment of plasmid pUX111 digested with the same restriction enzymes. pUX111 consists of the *Pst*I-*Hin*dIII restriction fragment of pUX-BF18 (28), which contains the *R. rubrum nifH* promoter region and the beginning of the *nifH* coding region replacing the *dra* promoter upstream of *draT* and a portion of *draT*, cloned into plasmid pUC19 (36).

Transformants from the ligation of the extension mixture to the pUX111 fragment were screened for the presence of plasmids containing the correct restriction digestion pattern expected for generation of the proper fusion of the *nifH* promoter and translation initiation region to the *draT* gene. One clone containing the correct fusion junction and surrounding sequence was designated pUX112. The *Bam*HI-*Hin*dIII restriction fragment of pUX112 containing this fusion was then cloned onto the mobilizable broad-host-range plasmid pRK404E1 (19), a derivative of plasmid pRK404 (3), to give pUX113. This plasmid was mobilized into *R. rubrum* (see below), and properly regulated overexpression of DRAT protein was demonstrated by immunoblot analysis of cell extracts from nitrogenase-repressed and -derepressed cultures of the resulting strain.

Because pUX113 is capable of overexpressing only functional DRAT, plasmids capable of overexpressing functional DRAT, DRAG, and the putative

product of the downstream ORF together or overexpressing only functional DRAG and the putative downstream ORF product were also generated. To construct a plasmid capable of expressing all three proteins, the *Bam*HI-*Sac*II restriction fragment from pUX112 containing the *nifH* promoter region fused to the beginning of *draT* was ligated to plasmid pUX109 digested with the same restriction enzymes. pUX109 consists of the *Pst*I fragment from pUX-BF18, containing the entire *dra* operon downstream of the *nifH* promoter region and beginning of the *nifH* coding region, cloned into pUC19. The joining of the pUX112 and pUX109 fragments placed the entire *dra* operon under the control of the *nifH* promoter region fused to *draT* in the resulting plasmid, pUX114. A plasmid capable of overexpressing only functional DRAG and the putative downstream ORF product was constructed by replacing the wild-type *Bbs*I restriction fragment from pUX114 with the *Bbs*I restriction fragment from pJHL220 containing the *draT3* mutation (21) to generate pUX125. This mutation is an in-frame insertion in the *draT* gene, replacing arginine 191 of DRAT with the amino acid sequence Glu-Phe-Pro-Gly-Ile and abolishing ADP-ribosylation of dinitrogenase reductase when present in the genome of *R. rubrum.*

The *Bam*HI-*Pst*I restriction fragments containing the *nifH* promoter region fused to the *dra* operons from plasmids pUX114 and pUX125 were also cloned into the mobilizable broad-host-range plasmid pRK404E1 to generate plasmids pUX115 and pUX126, respectively. A control plasmid, pUX116, was also generated in pRK404E1 by cloning into it the 480-bp *Bam*HI restriction fragment from pUX109 which contains the beginning of *nifH* and the *nifH* promoter region (19).

Bacterial strains and growth conditions. The *R. rubrum* strains were derived from matings between *E. coli* strains containing the mobilizable broad-host-range versions of the overexpression plasmids and *R. rubrum* UR2. UR2 (6) (genotype *str-1001*) is an otherwise wild-type streptomycin-resistant derivative of strain UR1, which is a laboratory-passaged derivative of strain ATCC 11170. *R. rubrum* strains were grown on SMN medium at 30°C (6, 17).

Transconjugant *R. rubrum* strains containing mobilizable broad-host-range versions of the overexpression plasmids are as follows: UR355 is UR2(pUX113) and overexpresses only functional DRAT; UR356 is UR2(pUX115) and overexpresses functional DRAT, DRAG, and presumably the putative product of the downstream ORF; UR357 is UR2(pUX116) and serves as the control strain; and UR360 is UR2(pUX126) and overexpresses an inactive DRAT protein as well as functional DRAG and presumably the putative product of the downstream ORF.

E. coli DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was used as the host strain for all plasmid-cloning experiments. Consequently, derivatives containing the mobilizable broad-host-range versions of the overexpressing plasmids served as donor strains in all matings to *R. rubrum*. Strain MM294A (pRK2013) (from Graham Walker, Massachusetts Institute of Technology) served as the donor of the pRK2013 helper plasmid in these matings (see below). All *E. coli* strains were grown aerobically with shaking in LC medium (17) at 37°C.

Antibiotics were used for selection of plasmids or as counterselective agents in matings as follows: ampicillin sodium salt, 100 μg/ml; kanamycin sulfate, 25 μg/ml; tetracycline hydrochloride, 1 μg/ml (*R. rubrum*) or 10 μg/ml (*E. coli*); streptomycin sulfate, 100 μg/ml.

Mating conditions. The mobilizable broad-host-range overexpression plasmid constructs were transferred into *R. rubrum* UR2 by conjugation in triparental matings with the helper plasmid pRK2013 (5). Donor and recipient cultures were grown to high turbidity with appropriate antibiotics under standard conditions. The *E. coli* donor strains were then subcultured by diluting 1:10 into medium lacking antibiotics and incubated under standard growth conditions for 2 h before use. Equal volumes, typically 100 μl, of the UR2 culture and of each donor and helper strain subculture were mixed in microcentrifuge tubes and collected by centrifugation for 30 s. The supernatant was discarded, the tubes were centrifuged for an additional 5 s, and the cell pellet was resuspended in the remaining liquid. This bacterial suspension was spread evenly over the surface of a 0.45-μm-pore-size 25-mm membrane filter (Gelman Sciences Inc., Ann Arbor, Mich.) on an SMN medium plate and incubated at 30°C aerobically in the dark for 24 h. The filters were then removed aseptically from the plates, and the cells were resuspended by vortexing in 1 ml of SMN medium. Dilutions were performed in SMN medium and were plated onto plates containing appropriate selective medium for determination of donor and recipient viable cell counts, as well as for frequency determinations for the mating event. Donor and recipient strains were also taken through the mating procedure separately as controls for these experiments.

Transfer of mobilizable broad-host-range overexpression plasmid constructs occurred at frequencies greater than 50% per recipient, and plasmid loss was estimated at less than 5% per generation. The ColE1-derived helper plasmid pRK2013 did not appear to replicate autonomously in *R. rubrum* UR2 following these matings.

Growth of overexpression strains and NH_4Cl or darkness treatment. Growth conditions were similar to those used by Kanemoto and Ludden (15). Cell cultures of *R. rubrum* were grown at 30°C in a 500-ml water-jacketed vessel containing malate-glutamate medium. Cultures were inoculated with a 15-ml starter culture to an A_{600} of approximately 0.13. Media for the overexpressing strains were supplemented with appropriate antibiotics. The culture was illuminated with a 150-W floodlight and continuously flushed with helium. Cell growth was monitored by measurement of light scattering at 600 nm. When the culture reached an A_{600} of approximately 1.4, switch-on/off experiments were performed as follows. The nitrogenase activity of whole cells was measured by the acetylene reduction method (2, 21). The light source was then removed, and the nitrogenase activity of culture samples was monitored. At the end of several light-dark cycles, a degassed solution of NH_4Cl was added to the culture so that the final concentration of NH_4^+ was 2 mM. After the final activity measurement and sample processing, the remaining culture cell paste was collected and stored in liquid nitrogen for DRAG-DRAT activity measurements and nitrogenase protein quantitation.

Immunoblot analysis of dinitrogenase reductase. To monitor the modification state of dinitrogenase reductase during switch-on/off conditions, culture samples were processed to view the protein by gel electrophoresis. At the end of each cycle, 1 ml of cell culture was withdrawn anaerobically from the vessel and injected into a 21-ml vial containing 1 ml of anaerobic sodium dodecyl sulfate (SDS) buffer. The SDS buffer contained 130 mM Tris (pH 6.8), 4.2% (wt/vol) SDS, 20% (vol/vol) glycerol, 0.003% (wt/vol) bromphenol blue, and 10% (vol/vol) 2-mercaptoethanol (added fresh). The vial was incubated at 30°C for 1 min and then boiled for 30 s. After 3 min of centrifugation, the supernatant was stored at −80°C. To obtain a more concentrated sample from dark cultures of UR356, 5 ml of cell culture was centrifuged anaerobically in the dark before breakage with 0.5 ml of SDS buffer. The SDS buffer breaks the cells and prepares the sample for analysis by gel electrophoresis.

Culture samples prepared as described above were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (18) with low-cross-linker gels (10% [wt/vol] total acrylamide; ratio of acrylamide to bisacrylamide, 172:1) which separate the ADP-ribosylated and unmodified subunits of dinitrogenase reductase (15). Proteins separated by SDS-PAGE were electrophoretically transferred onto a nitrocellulose membrane, which was then incubated with polyclonal antibodies against *R. rubrum* dinitrogenase reductase at a 1:5,000 dilution at 30°C for 1 h. The blot was then incubated with anti-rabbit immunoglobulin G alkaline phosphatase conjugate at a 1:8,000 dilution at 30°C for 40 min. Dinitrogenase reductase protein was visualized by alkaline phosphatase color development (1).

DRAG activity assays. Cells from the remaining culture were broken by sonication. The DRAG activity in these cell extracts was measured as previously described by incubating the DRAG source with reductant, dinitrogenase, and excess ADP-ribosylated dinitrogenase reductase in a nitrogenase assay mixture for 20 min (33). DRAG activity was monitored by coupling to the acetylene reduction activity of nitrogenase following cleavage of the ADP-ribose group of dinitrogenase reductase. DRAG activity is reported as nanomoles of ethylene produced per milligram of extract protein per minute.

DRAT activity assays. Crude extracts were prepared by sonication and tested for DRAT activity as previously described by incubating the DRAT source with active dinitrogenase reductase and [α-^{32}P]NAD for 20 min at 30°C (22). The proteins were then precipitated with 5% trichloroacetic acid and collected onto Gelman Sciences Metricel GN-6 membrane filters. The filters were washed several times with trichloroacetic acid, and the radioactivity on the filters was determined in a liquid scintillation counter. [α-^{32}P]NAD was prepared from [α-^{32}P]ATP and nicotinamide mononucleotide, using NAD-pyrophosphorylase (34). DRAT activity is reported as picomoles of ADP-ribose incorporated into dinitrogenase reductase per milligram of protein per minute.

ELISA quantitation of nitrogenase proteins. Dinitrogenase reductase or dinitrogenase proteins from crude extracts were quantitated by a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA). Flat-bottomed Falcon microtiter plates were prepared by coating each well (2 h, 30°C) with 50 μl of cell crude extract diluted in coating buffer (25) to a protein concentration of approximately 0.50 μg/ml. The wells were then blocked for 2 h at 30°C with 200 μl of 5% dry milk in phosphate-buffered saline (PBS)-Tween 20, incubated for 1.5 h at 30°C with 50 μl of polyclonal antibodies raised against *R. rubrum* dinitrogenase reductase or dinitrogenase at a 1:5,000 dilution in PBS–Tween 20–5% dry milk, and finally incubated for 1.5 h at 30°C with 50 μl of a 1:500 dilution of anti-rabbit immunoglobulin G alkaline phosphatase conjugate in PBS–Tween 20–5% dry milk. The reaction was developed with 100 μl of Sigma 104 phosphatase substrate (1 mg/ml) for 30 min and then stopped by the addition of 25 μl of 3 M NaOH. The wells were washed three times with PBS–0.05% (wt/vol) Tween 20 between each incubation step. The A_{410} was measured with a Dynatech MR700 Microplate Reader.

RESULTS

Generation of plasmid constructs overexpressing proteins from the *R. rubrum dra* region. Overexpression of proteins encoded by the *R. rubrum dra* operon was achieved by a perfect fusion of the *R. rubrum nifH* promoter and translation initiation sequences, including the initial ATG codon of *nifH*, to the *draT* gene at its second codon. This fusion was cloned into the *dra* operon in place of the *dra* promoter and wild-type *draT* translation initiation sequences to allow high-level expression of the entire operon under the control of the *nifH* promoter. Similar constructs containing a deletion of part of *draG* and all

TABLE 1. Characteristics of overexpressing strains

Strain	Protein overexpressed	DRAG activity[a]	DRAT activity[b]	Relative quantity (%) of protein[c]:		Initial nitrogenase activity[d]
				Dinitrogenase	Dinitrogenase reductase	
UR357	None (control)	16.64 (×1.0)	3.47 (×1.0)	17.5 (100%)	49.1 (100%)	840 (100%)
UR355	DRAT	19.85 (×1.2)	344 (×99)	9.30 (53%)	10.9 (22%)	60 (7.0%)
UR360	DRAG, ORF	2,055 (×124)	3.80 (×1.1)	17.3 (99%)	38.6 (78%)	510 (61%)
UR356	DRAT, DRAG, ORF	1,677 (×101)	1,182 (×341)	17.5 (100%)	43.5 (88%)	235 (28%)

[a] Expressed as nanomoles of ethylene produced per milligram of protein per minute. Values in parentheses compare the DRAG activity in overexpressing strains with the activity in UR357 (control strain).

[b] Expressed as picomoles of ADP-ribose incorporated into dinitrogenase reductase per milligram of protein per minute. Values in parentheses compare the DRAT activity in mutant strains with the activity in UR357 (control strain).

[c] The dinitrogenase or dinitrogenase reductase protein content in crude extracts of the indicated overexpressing strain was determined by ELISA and expressed as A_{410} per milligram of extract protein. The values in parentheses represent the quantity of dinitrogenase or dinitrogenase reductase in that strain compared with strain UR357 (control strain).

[d] Whole-cell nitrogenase activity expressed as nanomoles of ethylene formed per milliliter of culture per hour. The values in parentheses represent the activity as a percentage of the activity in strain UR357 (control strain).

of the downstream ORF, to allow expression of DRAT only, or containing a nonpolar mutation in *draT*, to allow expression only of functional DRAG and the putative downstream ORF-encoded protein, were also created.

All of these constructs were subsequently cloned onto the broad-host-range plasmid pRK404E1 (19) and introduced into wild-type *R. rubrum*. A control plasmid containing the wild-type *nifH* promoter region and the beginning of the *nifH* coding region cloned onto pRK404E1, without any of the *dra* coding region, was also introduced into wild-type *R. rubrum* to examine possible effects on nitrogenase regulation that are due solely to the presence of pRK404E1 or multiple copies of the *nifH* promoter.

Properly *nif*-regulated overexpression of DRAT and/or DRAG from the appropriate constructs in *R. rubrum* was observed following immunoblot analysis of crude cell extracts. No overexpression of DRAT or DRAG proteins was detectable in strain UR357 (control strain). Interestingly, the strain containing the overexpression plasmid with the nonpolar *draT* mutation (UR360) still accumulated overexpressed DRAT protein (data not shown), although this protein lacked ADP-ribosyltransferase activity (see below). Overexpression of the putative downstream ORF-encoded protein from the appropriate plasmids was not examined, since this protein has yet to be identified by gel electrophoresis and since antiserum that recognizes it has not yet been generated.

Levels of DRAT and/or DRAG in constructed strains. The in vitro DRAT and DRAG activities in strains UR355 (DRAT overexpresser), UR356 (DRAT-DRAG-ORF overexpresser), and UR360 (DRAG-ORF overexpresser) were determined and compared with the activities in strain UR357 (control strain). As seen in Table 1, UR356 and UR360 both contain at least 100 times as much DRAG activity as UR357 does. UR355 contains 100 times more DRAT activity than UR357 does, and UR356 contains 340 times more DRAT activity than UR357 does. Strains UR360 and UR356 contain plasmids which should also overexpress the product of the downstream ORF. Overexpression of this downstream ORF was not confirmed, because an assay for the protein has not been identified.

Immunoblot analysis showed that an overexpressed DRAT polypeptide accumulated in strain UR360 (see above), but in vitro DRAT activity assays showed that this accumulated form did not ADP-ribosylate dinitrogenase reductase. Strain UR360 does contain wild-type quantities of active DRAT (Table 1), because of the placement of the constructed plasmids in an *R. rubrum* wild-type background.

Nitrogenase regulation is not altered in the plasmid control strain. In wild-type strains of *R. rubrum*, the whole-cell nitrogenase activity decreases upon exposure of cells to darkness or ammonium (21). ADP-ribosylation of dinitrogenase reductase, catalyzed by the DRAT enzyme, causes this activity decrease (15). To examine if the presence of multiple plasmid-borne copies of the *nifH* promoter region affects nitrogenase regulation, UR357 (control strain) was studied during light-dark cycles or following addition of ammonium chloride.

As shown in Fig. 2A, in a UR357 (control strain) culture the nitrogenase activity decreased to 31% of its initial activity after a 40-min exposure to darkness. The cells rapidly recovered activity when the culture was reilluminated. The nitrogenase activity also declined when the culture was exposed to 2 mM

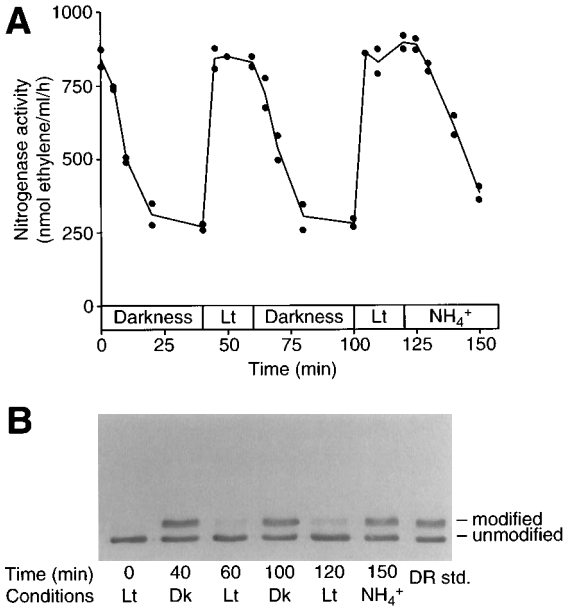

FIG. 2. Response of the nitrogenase enzyme in *R. rubrum* UR357 (plasmid control strain) to inactivating conditions. Strain UR357 was grown photosynthetically and derepressed for nitrogenase. (A) The whole-cell nitrogenase activity was measured in duplicate at the indicated times. The culture was exposed to two cycles of 40 min of darkness (Dk) and 20 min of reillumination (Lt), followed by addition of NH_4Cl to a final concentration of 2 mM. (B) Immunoblot analysis of dinitrogenase reductase from samples withdrawn from the culture at the indicated times and culture conditions and processed as described in Materials and Methods. Lane "DR std." contains a fully modified dinitrogenase reductase standard sample.

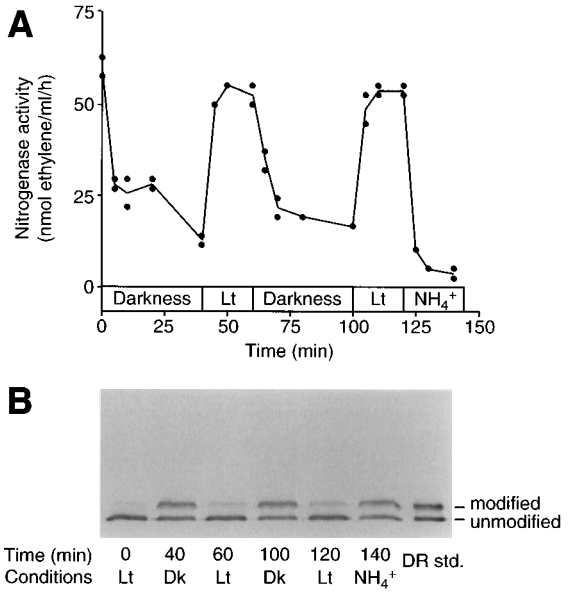

FIG. 3. Response of the nitrogenase enzyme in *R. rubrum* UR355 (DRAT overexpresser) to inactivation conditions. See the legend to Fig. 2 for experimental details. (A) Response of nitrogenase activity to dark, light, and ammonium exposure. (B) Corresponding immunoblot analysis of the dinitrogenase reductase protein.

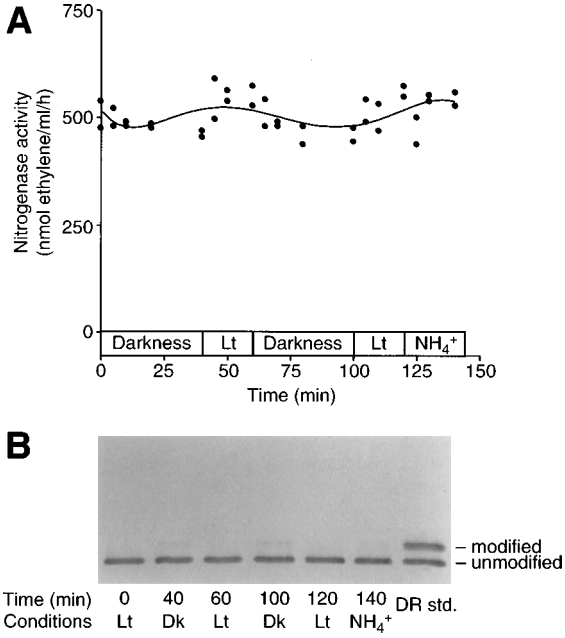

FIG. 4. Response of the nitrogenase enzyme in *R. rubrum* UR360 (DRAG-ORF overexpresser) to inactivation conditions. See the legend to Fig. 2 for experimental details. (A) Response of nitrogenase activity to dark, light, and ammonium exposure. (B) Corresponding immunoblot analysis of the dinitrogenase reductase protein.

ammonium chloride. Immunoblot analysis of the dinitrogenase reductase protein (Fig. 2B) showed that when the culture was exposed to light (0, 60, and 120 min), the protein was in an unmodified state, as seen by the appearance of only the lower (unmodified) dinitrogenase reductase band upon electrophoresis. However, in samples taken from the culture exposed to darkness or ammonium, dinitrogenase reductase appeared as two bands that represent the modified (upper band) and unmodified (lower band) subunits of the protein. This switch-on/off experiment shows that UR357 exhibits wild-type post-translational regulation of nitrogenase, indicating that the plasmid vector and multiple copies of the promoter have no obvious effects on nitrogenase regulation.

DRAT overexpression does not alter nitrogenase regulation. A similar switch-on/off experiment to that described above was performed with strain UR355 (DRAT overexpresser) to determine if overexpression of DRAT by itself altered nitrogenase regulation. Figure 3A depicts the change in nitrogenase activity in response to dark-light cycles and ammonium. Similar to the control strain, in UR355 the nitrogenase activity declined when the culture was exposed to darkness and recovered when the culture was reilluminated. As seen in Fig. 3B, the decline in activity was due to modification of dinitrogenase reductase. When the UR355 culture was exposed to 2 mM ammonium chloride, the nitrogenase activity decreased to background levels within 10 min. This decrease in activity is notably more rapid than in UR357 (the control strain). Again, this decline in nitrogenase activity corresponds to modification of dinitrogenase reductase (Fig. 3B, lane 6). DRAT is apparently not active in UR355 during the initial light exposure and does not become active until the culture is exposed to darkness, similar to previous observations with normally expressed DRAT in wild-type strains (21). Regulation of DRAT activity in UR355 is similar to regulation in the control strain, indicating that the 99-fold increase in DRAT does not significantly alter its ability to be regulated.

The initial nitrogenase activity in UR355, at 0 min, is only 60 nmol/ml/h, which is 14 times less than the 840-nmol/ml/h nitrogenase activity in UR357 (the control strain). Immunoblot analysis shows that dinitrogenase reductase is not substantially modified at this point (Fig. 3B, lane 1), and therefore ADP-ribosylation of dinitrogenase reductase is not the cause of the low initial nitrogenase activity. The possible causes of this low activity are addressed below.

DRAG and ORF overexpression does alter nitrogenase regulation. Strain UR360 (DRAG-ORF overexpresser) was tested to determine if overexpression of DRAG alters nitrogenase regulation. Figure 4A shows the change in nitrogenase activity in response to dark-light cycles and ammonium. No significant decrease in nitrogenase activity was detected when the culture was exposed to darkness or 2 mM ammonium chloride. Correspondingly, only very small amounts of modified dinitrogenase reductase are detected by immunoblot analysis (Fig. 4B). Because this strain has wild-type levels of DRAT from the chromosomal copy, failure to see substantial modification in the dark or after exposure to ammonium indicates that sufficient DRAG remains active under these conditions and reverses the effect of active DRAT. This indicates that overexpression of DRAG alters the ability of the cell to normally regulate DRAG activity.

Overexpression of DRAT, DRAG, and ORF does not alter nitrogenase regulation. Strain UR356, which overexpresses DRAT, DRAG, and the downstream ORF, was tested for nitrogenase regulation in dark-light cycles and after exposure to ammonium. Nitrogenase activity decreased in the dark and in the presence of ammonium because of ADP-ribosylation of dinitrogenase reductase (Fig. 5). Although DRAG is overexpressed in this strain, as in UR360 (DRAG-ORF overexpresser), it now appears to become inactive in the dark or during exposure to ammonium, allowing for proper ADP-ribosylation of dinitrogenase reductase by DRAT. The nitroge-

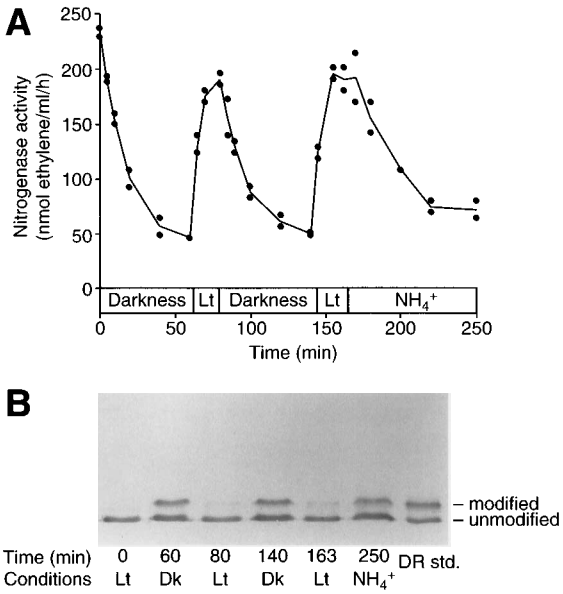

FIG. 5. Response of the nitrogenase enzyme in *R. rubrum* UR356 (DRAG-DRAT-ORF overexpresser) to inactivation conditions. See the legend to Fig. 2 for experimental details. (A) Response of nitrogenase activity to dark, light, and ammonium exposure. (B) Corresponding immunoblot analysis of the dinitrogenase reductase protein.

nase activity recovered to its original level upon culture reillumination (Fig. 5), indicating that overexpressed DRAG is showing complete regulation by being inactivated in the dark and in the presence of ammonium and being activated in the light.

Typically, the intensity of the two dinitrogenase reductase bands is equal for samples with low nitrogenase activity due to ADP-ribosylation (32). However, the upper band from dark culture samples of strain UR356 has approximately 60% of the intensity of the lower band (Fig. 5B) as determined by densitometry scanning. We suspect that the ADP-ribose bond to dinitrogenase reductase is cleaved by the excess DRAG upon cell lysis.

Strains that overexpress DRAT have lower initial nitrogenase activity. Cultures of these overexpressing strains were grown to an A_{600} of 1.4, at which time the whole-cell nitrogenase activity was measured. This activity varied among strains: UR357 (control), 840 nmol/ml/h; UR355 (DRAT overexpresser), 60 nmol/ml/h; UR360 (DRAG-ORF overexpresser), 510 nmol/ml/h; and UR356 (DRAT-DRAG-ORF overexpresser), 235 nmol/ml/h. The cause of reproducibly low initial nitrogenase activity in several of these strains was explored. As stated above, the low initial activities of strains UR355 and UR356 are not due to ADP-ribosylation of dinitrogenase reductase, as evidenced by the absence of an upper, modified subunit band in illuminated culture samples.

Since nitrogenase activity is dependent on both dinitrogenase and dinitrogenase reductase, the quantities of these proteins in the overexpressing strains were measured in crude extracts by ELISA and compared with the quantities in the control strain (Table 1). Strains UR357, UR360, and UR356 all contain approximately the same quantity of the nitrogenase proteins as measured by ELISA. Therefore, the more-than-threefold-lower initial nitrogenase activity in strain UR356 compared with UR357 (the control strain) is not due to a smaller quantity of nitrogenase proteins. Furthermore, strain UR355 contains fourfold less dinitrogenase reductase protein and twofold less dinitrogenase protein than does strain UR357. However, these lower protein contents cannot completely explain the 14-fold-lower initial nitrogenase activity in strain UR355 than in strain UR357. Another possible cause of the low initial activity will be discussed below.

DISCUSSION

In wild-type *R. rubrum*, DRAT is activated in the dark or in the presence of ammonium and DRAG is activated in the presence of light and inactivated in the dark or in the presence of ammonium. How these conditions are communicated to DRAT and DRAG is unknown. Although posttranslational regulation of DRAT and DRAG activities has been demonstrated in vivo, it has yet to be demonstrated in vitro since this regulation is lost upon cell breakage. The differences between active and inactive DRAT and/or DRAG are unknown. By studying DRAT and DRAG regulation in *R. rubrum* strains that overexpress these regulatory enzymes, further information on their regulation has been obtained.

In the *R. rubrum* strain UR355 (DRAT overexpresser), nitrogenase regulation and thus DRAT regulation appears not to be altered. DRAT appears not to be active until the cells are exposed to darkness or ammonium, as shown by the presence of only unmodified dinitrogenase reductase in an illuminated culture before exposure to darkness or ammonium. There is no way of knowing what portion of overexpressed DRAT is activated by darkness or ammonium, since the only way to assay DRAT activity in vivo is indirectly via nitrogenase inactivation. However, DRAT is regulated, and thus there is sufficient regulatory molecule present to control the overexpressed DRAT.

In contrast to the proper regulation of overexpressed DRAT, DRAG does not appear to be properly regulated in strain UR360 (DRAG-ORF overexpresser). Strain UR360 displays the phenotype of a *draT* mutant in which nitrogenase activity does not decrease in response to darkness or addition of ammonium (21). Because of the single copy of the *draT* gene in the chromosome, wild-type DRAT activity is present in UR360 crude extracts; however, no significant decrease of nitrogenase activity in response to darkness or ammonium is seen. There are two possible models to explain this lack of dinitrogenase reductase inactivation in UR360. (i) When DRAG is overexpressed, some of it remains active in darkness and in the presence of ammonium and thereby reactivates dinitrogenase reductase as soon as DRAT inactivates it. In this model a molecule required for proper DRAG regulation is limiting, and thus some of the DRAG escapes regulation. (ii) The lack of dinitrogenase reductase inactivation in strain UR360 is due to the overexpression of DRAG altering DRAT activity or regulation such that it no longer catalyzes the inactivation of dinitrogenase reductase by ADP-ribosylation. We do not favor the second explanation, because in processed samples from a dark- or ammonium-treated UR360 culture, there is a very faint upper band representing ADP-ribosylated dinitrogenase reductase. This faint band is not detected in processed samples taken from an illuminated culture. This suggests that DRAT is still active and properly regulated in vivo and that the lack of nitrogenase inactivation in strain UR360 is due to disruption of DRAG regulation.

If in strain UR360 (DRAG-ORF overexpresser) nitrogenase regulation is altered by incomplete DRAG inactivation in the dark or in the presence of ammonium, it is surprising that regulation is not altered in strain UR356, which also overexpresses DRAG and the downstream ORF, in addition to DRAT. Two possible ways to interpret DRAG regulation in strain UR356 are as follows: (i) while overexpressed DRAG remains active under conditions of darkness or ammonium, the overexpressed DRAT is activated, outcompetes DRAG, and

modifies dinitrogenase reductase; or (ii) DRAG is now inactivated in the dark or in the presence of ammonium, leading to proper nitrogenase regulation. We believe that DRAG is being inactivated in this strain, because of the following argument. Our current model for nitrogenase regulation proposes that DRAT is transiently activated following exposure of cells to negative stimuli. DRAT ADP-ribosylates dinitrogenase reductase but then is inactivated by the time of the nitrogenase activity minimum (37). If this model is correct, the overexpressed DRAG in UR356 must be inactive at the nitrogenase activity minimum, where DRAT is no longer active, or an increase in nitrogenase activity would be observed before reillumination of the culture. Since no increase is observed, overexpressed DRAG must be displaying proper regulation when it is inactivated in the dark or in the presence of ammonium and activated in the light.

As stated above, the only difference between strain UR356, in which overexpressed DRAG is properly regulated, and strain UR360, in which overexpressed DRAG is not properly regulated, is that strain UR356 also overexpresses DRAT. Therefore, we believe that a proper balance between DRAT and DRAG is necessary for negative regulation of overexpressed DRAG activity. The direct or indirect effect of DRAT on DRAG regulation will be the focus of further study.

That DRAT is required for DRAG inactivation is consistent with the following observation. Nitrogenase inactivation after ammonium addition is much more rapid in a *draG* mutant than in the wild type (21), suggesting that the rate of nitrogenase inactivation is dependent on the rate of DRAG inactivation in the presence of ammonium. If DRAT is necessary for DRAG inactivation, as our data suggest, then, in a strain overexpressing DRAT, DRAG inactivation and thus nitrogenase inactivation should be very rapid, as is seen in strain UR355 (DRAT overexpresser) (Fig. 3). Further support for interaction of DRAT and DRAG in vivo is that DRAT accumulates to a greater extent in strain UR356 (DRAT-DRAG-ORF overexpresser) than strain UR355 (DRAT overexpresser). This increased accumulation may be due to a DRAG-DRAT interaction which increases DRAT stability.

Strains that overexpress functional DRAT have a low initial whole-cell nitrogenase activity. This low activity is not due to dinitrogenase reductase modification or solely to the presence of smaller quantities of the nitrogenase proteins in these strains. Another possible explanation being explored is that DRAT interacts with dinitrogenase reductase in such a way that the electron transfer between dinitrogenase reductase and dinitrogenase is interrupted. This interference seems plausible, since the site of ADP-ribosylation on dinitrogenase reductase is very near the proposed site of contact with dinitrogenase (35). DRAT must have some affinity for dinitrogenase reductase, and when DRAT is overexpressed this interaction may occur, leading to disruption of dinitrogenase reductase activity.

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