

## Effect of Perturbation of ATP Level on the Activity and Regulation of Nitrogenase in *Rhodospirillum rubrum*<sup>∇</sup>

Yaoping Zhang, Edward L. Pohlmann, and Gary P. Roberts\*

Department of Bacteriology, University of Wisconsin-Madison, Madison, Wisconsin 53706

Received 5 May 2009/Accepted 15 June 2009

**Nitrogenase activity in *Rhodospirillum rubrum* and in some other photosynthetic bacteria is regulated in part by the availability of light. This regulation is through a posttranslational modification system that is itself regulated by P<sub>II</sub> homologs in the cell. P<sub>II</sub> is one of the most broadly distributed regulatory proteins in nature and directly or indirectly senses nitrogen and carbon signals in the cell. However, its possible role in responding to light availability remains unclear. Because P<sub>II</sub> binds ATP, we tested the hypothesis that removal of light would affect P<sub>II</sub> by changing intracellular ATP levels, and this in turn would affect the regulation of nitrogenase activity. This in vivo test involved a variety of different methods for the measurement of ATP, as well as the deliberate perturbation of intracellular ATP levels by chemical and genetic means. To our surprise, we found fairly normal levels of nitrogenase activity and posttranslational regulation of nitrogenase even under conditions of drastically reduced ATP levels. This indicates that low ATP levels have no more than a modest impact on the P<sub>II</sub>-mediated regulation of NifA activity and on the posttranslational regulation of nitrogenase activity. The relatively high nitrogenase activity also shows that the ATP-dependent electron flux from dinitrogenase reductase to dinitrogenase is also surprisingly insensitive to a depleted ATP level. These in vivo results disprove the simple model of ATP as the key energy signal to P<sub>II</sub> under these conditions. We currently suppose that the ratio of ADP/ATP might be the relevant signal, as suggested by a number of recent in vitro analyses.**

The P<sub>II</sub> family of regulatory proteins is one of the most broadly distributed in nature, being found in all three domains of life (1, 20, 39, 51). P<sub>II</sub> was originally found to be involved in the regulation of glutamine synthetase (GS) activity by interaction with adenyltransferase (ATase or GlnE, encoded by *glnE*) (63, 65). However, P<sub>II</sub> has subsequently been shown to regulate many other processes involving nitrogen assimilation and metabolism, by directly interacting with different proteins, termed P<sub>II</sub> receptors (51). In many organisms, more than one P<sub>II</sub> homolog has been identified. The “housekeeping” homolog is usually referred to as GlnB, encoded by *glnB*, with other secondary homologs named GlnK, GlnK<sub>2</sub>, GlnJ, GlnY, GlnZ, or Pz (1, 12, 47, 69, 78). In a few species of *Archaea*, a distinct group of P<sub>II</sub> proteins was named NifI, which is encoded by the *nifH*-linked gene (39).

Because of its physiological diversity, the photosynthetic bacterium *Rhodospirillum rubrum* is a good model organism in which to study the role of P<sub>II</sub>. It has three P<sub>II</sub> homologs—GlnB, GlnK, and GlnJ—that play distinct and overlapping functions by interacting with at least six receptors in the cell (71, 78, 81). Some of these are common to other bacteria, such as GlnE (ATase) that is involved in the regulation for GS activity, the two-component regulatory protein NtrB, and the ammonium transporter (gas channel) AmtB<sub>1</sub> (71, 78). Other P<sub>II</sub> receptors in *R. rubrum* are involved in nitrogen fixation: NifA is the transcriptional activator for nitrogen fixation genes,

and two additional proteins perform posttranslational regulation of nitrogenase activity (78–80).

In *Klebsiella pneumoniae* and *Azotobacter vinelandii* NifA activity is regulated by direct interaction with NifL, another *nif* gene product, and P<sub>II</sub> is involved in the NifA-NifL interaction (24, 29, 44, 45). However, in *R. rubrum*, NifA activity requires direct interaction with the uridylylated form of GlnB, and neither GlnK nor GlnJ can activate NifA (81). A similar regulation of NifA activity by GlnB has been reported in *Azospirillum brasilense*, *Rhodobacter capsulatus*, and *Herbaspirillum seropedicae* (2, 4, 18).

The posttranslational regulation of nitrogenase activity in *R. rubrum* involves the reversible mono-ADP-ribosylation of dinitrogenase reductase, the terminal electron donor to dinitrogenase (53, 80). Two enzymes perform this reversible reaction. Dinitrogenase reductase ADP-ribosyl transferase (referred to as DRAT, the gene product of *draT*) carries out the transfer of the ADP-ribose from NAD to one subunit of the dinitrogenase reductase homodimer, resulting in the inactivation of that enzyme. The ADP-ribose group attached to dinitrogenase reductase can be removed by another enzyme, dinitrogenase reductase activating glycohydrolase (referred to as DRAG, the gene product of *draG*), thus restoring nitrogenase activity. This regulatory system has also been found in *A. brasilense*, *Azospirillum lipoferum*, *R. capsulatus*, and a few other nitrogen-fixing bacteria (21, 48, 49, 74). It negatively regulates nitrogenase activity in response to exogenous NH<sub>4</sub><sup>+</sup> or to energy limitation in the form of dark shift (in the cases of *R. rubrum* and *R. capsulatus*) or anaerobiosis shifts (in *A. brasilense*) (41, 48, 73). The regulation of the ADP-ribosylation of dinitrogenase reductase is effected through the posttranslational regulation of

\* Corresponding author. Mailing address: Department of Bacteriology, University of Wisconsin-Madison, Madison, WI 53706. Phone: (608) 262-3567. Fax: (608) 262-9865. E-mail: groberts@bact.wisc.edu.

<sup>∇</sup> Published ahead of print on 19 June 2009.

both DRAT and DRAG activities, and  $P_{II}$  is involved in the regulation of both enzymes (25, 27, 28, 67, 70, 77, 78).

The activity of  $P_{II}$  and its interaction with different receptors is mainly dependent on the conformation of  $P_{II}$  in the cell.  $P_{II}$  exists in multiple forms, depending on the carbon and nitrogen levels in the cell. The conformational change of  $P_{II}$  is caused by its direct binding of small molecules such as 2-oxoglutarate or  $\alpha$ -KG (as a signal of carbon excess) and ADP/ATP, and indirectly by its covalent modification of the protein in response to nitrogen status (16, 17, 31–34, 51, 66, 71). In many bacteria,  $P_{II}$  is modified by uridylylation, which is carried out by a bifunctional, uridylyltransferase/uridylyl-removing enzyme (UTase/UR). This enzyme is encoded by *glnD* and senses the intracellular concentration of glutamine in the cell (32). Through the action of UTase/UR the uridylylated form of  $P_{II}$  signals a nitrogen deficiency.

Because the DRAT/DRAG system responds to changes in energy and has been shown to be regulated by  $P_{II}$ , it was a plausible hypothesis that  $P_{II}$  itself senses the status of energy in some way (78, 82). For example, the DRAT/DRAG regulatory system in *R. rubrum* mutants lacking GlnB and GlnJ failed to lower nitrogenase activity in response to a removal of light (78). Similarly, AmtB<sub>1</sub> sequesters  $P_{II}$  under conditions of nitrogen excess, and the mutation of *amtB<sub>1</sub>* in *R. rubrum* eliminated the regulation of nitrogenase activity not only in response to  $NH_4^+$  addition, but also in response to a shift to darkness (82). Given the clear role of ATP binding on  $P_{II}$  function in vitro (51), the level of ATP was an obvious candidate for the energy signal sensed by  $P_{II}$  in vivo. However, previous studies by two different groups showed different conclusions. Li et al. reported that the pool of ATP in *R. rubrum* decreased rapidly following the dark shift and then recovered slowly and partially. The ATP pool fully recovered after the cells were returned to light (40). In contrast, using a different extraction and detection method, Paul and Ludden saw that the pool of ATP decreased slightly and then increased rapidly within 30 s of a dark shift. These authors also reported further ATP fluctuations during the incubation in the dark, but overall the pool of ATP remained high (56). Recently, a variety of in vitro analysis have indicated that the ratio of ADP to ATP might be critical for a number of  $P_{II}$  functions (26, 31, 66, 71). Nevertheless, there has been no direct in vivo test of the impact of changes in internal ATP levels on  $P_{II}$  function, and the focus of the present study was to measure that impact by monitoring the DRAT/DRAG system.

ATP plays many critical roles in living cells (3, 7, 11, 13), and there are clearly cases where absolute ATP level is also an important indicator of the physiological state in prokaryotes (54, 55, 59, 60). Schneider et al. reported that the changes in ATP concentration affect the transcription of the *rrnB* P1 promoter (59). These authors perturbed the internal ATP levels through the use of a *purE* mutant, which has an impaired purine synthesis pathway, and showed that a six-fold-elevated ATP level correlated with high *rrnB* P1 promoter activity (59).

However, there are three main challenges in attempting to correlate ATP levels with physiological phenomena. (i) The metabolism of the cell must be stopped rapidly so that the ATP pool is not altered during processing. (ii) ATP exists in association with many proteins in the cell, and it is impossible to

know which fraction of the ATP is liberated by different extraction methods. (iii) Finally, it is impossible to know which fraction of this variously bound ATP pool is relevant for any single ATP-binding protein.

Many ATP extraction methods have been developed and tested in *Escherichia coli* and other bacteria, but these have produced dramatically different results (37, 46, 60). Even a single method performs differently when applied to different organisms (37, 46). An alternative technique to measure ATP involves the injection of firefly luciferase into mammalian cells (5, 36). The luciferase reporter system has also been used for monitoring ATP in the intact cells of *E. coli* and *R. capsulatus* (10, 14, 22, 60). However, this method has some challenges for its use. The substrate luciferin must be rapidly introduced into the cell, and this has typically demanded that the cells be treated with a permeabilizing drug such as polymyxin B (10, 14, 60). Such permeabilization almost certainly perturbs the ATP pool to some extent and prevents monitoring rapid changes in that pool. Another problem is that wild-type luciferase has a very low  $K_m$  for ATP and is therefore saturated even if ATP levels fluctuate (14). However, Schneider et al. studied the change in ATP concentration and its relationship with regulation of rRNA expression in *E. coli* by using a variant of luciferase (with an H245F substitution) that raises the enzyme's  $K_m$  for ATP to a physiologically reasonable level (6, 60).

We demonstrate here the technical problems with different in vitro ATP assays and then show that a luciferase reporter system in *R. rubrum* coincidentally avoids many of these problems and allows us to monitor the change of ATP concentration in the cells after dark-light shifts or the addition of  $NH_4^+$ . We then use *purE* mutants in *R. rubrum* to perturb intracellular ATP levels deliberately by providing different exogenous adenine levels. In the presence of limited concentrations of adenine, the ATP concentration was dramatically decreased in *purE* mutants, but the effects on nitrogenase activity and its regulation were surprisingly small; only the recovery of nitrogenase activity after the restoration of light was strongly affected. The modification state of GlnB was also altered under these conditions, a finding consistent with the model that  $P_{II}$  senses energy status in some way and then regulates DRAT/DRAG activity. Based on these results and a number of recent biochemical analyses that indicate the importance of the ADP/ATP ratio for the regulation of  $P_{II}$  functions in vitro (26, 31, 66, 71), we suggest that the important impact of light and dark in vivo might be on the ADP pool, which then affects that ratio. The results also suggest that the ATP-dependent electron transfer from dinitrogenase reductase to dinitrogenase is surprisingly insensitive to severely decreased ATP levels.

#### MATERIALS AND METHODS

**Growth conditions and in vivo nitrogenase activity assay.** *E. coli* was grown in LC medium (similar to Luria-Bertani medium, but with 5 g of NaCl/liter). *R. rubrum* was grown in yeast extract-supplemented malate- $NH_4^+$  (SMN) rich medium, malate-glutamate (MG) medium, or nitrogen-free (MN<sup>-</sup>) malate medium as described previously (19, 38, 41). The whole-cell nitrogenase activity assay and the methods of dark/light shifts and  $NH_4^+$  treatments have been described previously (72). Antibiotics were used as necessary at the levels described previously (79).

**ATP extraction and assay.** Four different extraction methods were compared for their effectiveness in extracting ATP from *R. rubrum* grown in MG medium involving the use of boiling, trichloroacetic acid (TCA), formic acid, or formal-

dehyde. The boiling and TCA methods were based on previously published methods (46) with some modifications. For the TCA method, 0.2 ml of *R. rubrum* culture was quickly mixed with 0.2 ml of 10% TCA and 4 mM EDTA solution. After incubation on ice for 10 min, the extract was centrifuged at  $20,000 \times g$  for 10 min, and the supernatant was saved at  $-80^\circ\text{C}$  for the ATP assay. For the boiling method, 0.1 ml of culture was mixed with 0.9 ml of preboiling Tris-EDTA buffer (0.1 M Tris and 2 mM EDTA with pH at 7.75), boiled for 2 min at  $100^\circ\text{C}$ , and incubated on ice for 10 min, and the extract was centrifuged at  $20,000 \times g$  for 10 min. The supernatant was saved at  $-80^\circ\text{C}$  for the ATP assay. The formic acid method was performed according to a published method (30) with the following modification: 0.5 ml of culture was quickly mixed with 0.1 ml of 2 M formic acid. After incubation on ice for 30 min, the extract was centrifuged at  $20,000 \times g$  for 10 min, and the supernatant was saved for the ATP assay. The formaldehyde method was based on the published method (43) except that 1 ml of culture was quickly mixed with 0.1 ml of 1.9% formaldehyde. After incubation on ice for 10 min, the extract was centrifuged at  $20,000 \times g$  for 10 min, and the supernatant was discarded. Pellets were dissolved in 50  $\mu\text{l}$  of 0.1 M KOH by vortexing and incubated on ice for 30 min, and then 50  $\mu\text{l}$  of 8.8%  $\text{H}_3\text{PO}_4$  was added to neutralize the KOH. The extract was centrifuged again at  $20,000 \times g$  for 10 min, and the supernatant was saved at  $-80^\circ\text{C}$  for the ATP assay.

The ENLITEN ATP assay system bioluminescence detection kit (Promega, Madison, WI) was used for the ATP assay. Before carrying out the assay, samples from TCA, formic acid, and formaldehyde extraction were diluted 100-fold with Tris-EDTA buffer, and the boiled samples were diluted 10-fold in the same buffer. The luminescence was measured with a TD-20/20 luminometer (Turner Biosystems, Sunnyvale, CA) with a 2-s delay period and a 10-s integration period. The ATP concentration was expressed as nmol per mg of dry weight, based on previous data that the dry weight of *R. rubrum* was  $0.28 \pm 0.01$  mg per ml of culture at an optical density at 600 nm ( $\text{OD}_{600}$ ) of 1 (76).

**Expression of luciferase in *R. rubrum* and assay of ATP concentration in intact cells.** The luciferase expression vector was constructed as follows. The promoter region of *aacC1* (gentamicin resistance [ $\text{Gm}^r$ ] gene) from pUCGM (62) was first amplified by PCR and cloned into pBSKS(-) (Stratagene, La Jolla, CA), yielding pUX2519. An NdeI site was created at the first methionine codon of *aacC1*. The structural gene encoding *Photinus pyralis* luciferase with the H245F substitution was then amplified from a pGEX derivative (60) (kindly provided by R. Gourse) and subcloned into pUX2519, yielding pUX2523. Similarly, an NdeI site was created at the start codon of the luciferase gene so that it could be ligated perfectly to the *aacC1* promoter. The fragment carrying the region encoding the luciferase-H245F linked with the promoter of *aacC1* was then cloned into pRK404 (15), yielding pUX2524, which was transferred into *R. rubrum* by a standard triparental mating procedure as described previously (23).

The ATP assay in intact cells of *R. rubrum* was done in an assay buffer containing 10 mM HEPES (pH 6.8), 150 mM NaCl, 0.6 mM KCl, 10 mM  $\text{MgSO}_4$ , 0.2% glucose, and 4 mM luciferin unless indicated otherwise. Polymyxin B was only used in the initial experiments and omitted in the routine assays because it was unnecessary for the assay with *R. rubrum* cells. Then, 10  $\mu\text{l}$  of *R. rubrum* cells was mixed with 100  $\mu\text{l}$  of assay buffer by brief vortexing, and the relative light units (RLU) were measured with a TD-20/20 luminometer with a 2-s delay period and a 2-min integration period. The ATP assay was done in a 12-by-15-mm tube under aerobic conditions, since oxygen is necessary for luciferase activity. Control experiments showed that the RLU were constant in the range of 30 s to 15 min (data not shown). In vivo ATP concentration is expressed as RLU per mg of dry weight.

**Cloning and construction of *purE* mutant from *R. rubrum*.** The sequence of the *purE* homolog of *R. rubrum* was obtained from the genomic sequence of *R. rubrum*, which is available from the U.S. Department of Energy Joint Genome Institute ([http://genome.jgi-psf.org/finished\\_microbes/rhoru/rhoru.home.html](http://genome.jgi-psf.org/finished_microbes/rhoru/rhoru.home.html)). A 4-kb fragment of the *purEK* region was amplified from the genomic DNA of wild-type *R. rubrum* using *Pfu* DNA polymerase (Stratagene) and a pair of oligonucleotide primers with StuI and PstI sites at their ends. After digestion with StuI and PstI, the amplified DNA fragment was cloned into pUX19 (42), yielding pUX2112, which was confirmed by sequencing.

To construct the *purE* insertion mutants, pUX2112 was digested with EcoRV, which cuts *purE* at codon 143, and a  $\text{Gm}^r$  cartridge from pUCGM (62) was inserted in either orientation, yielding pUX2362 and pUX2363. Both pUX2362 and pUX2363 were digested with PvuII, and the fragments containing *pruE::aacC1* were cloned into pSUP202 (64), yielding pUX2364 and pUX2365, respectively. These plasmids were transformed into *E. coli* S17-1 (64) and then conjugated into *R. rubrum* wild-type (UR2) by a previously described method (41). Streptomycin-resistant  $\text{Gm}^r$  *R. rubrum* colonies were selected and replica printed to identify chloramphenicol-sensitive colonies resulting from a double-crossover recombination event. In UR2187 (*purE1::aacC1*) *purE* and *aacC1* are

TABLE 1. Comparison of ATP levels in MG-grown *R. rubrum* wild type (UR2) with different extraction methods

Method	Mean ATP concn (nmol/mg of dry wt) $\pm$ SD
TCA extraction.....	5.29 $\pm$ 0.39
Formic acid extraction.....	4.57 $\pm$ 0.43
Formaldehyde extraction.....	0.93 $\pm$ 0.14
Boiling .....	2.50 $\pm$ 0.29

transcribed in the same direction, whereas in UR2188 (*purE2::aacC1*) they are transcribed in the opposite direction.

**Generating GlnB antiserum and immunoblotting procedures.** *R. rubrum* GlnB was overexpressed and purified as described previously (83). For generation of *R. rubrum* GlnB antiserum, a rabbit was immunized by intradermal injection of 1 mg of pure *R. rubrum* GlnB protein with complete Freund adjuvant and then with two subsequent boosts. The serum was collected at 4 weeks after the final boost.

As described previously, a TCA precipitation method was used to extract proteins, and a low cross-linker (ratio of acrylamide-bisacrylamide, 172/1) Tricine gel (16.5%) was used to separate the modified and unmodified  $\text{P}_{\text{II}}$  (58, 82). Proteins obtained from sodium dodecyl sulfate-polyacrylamide gel electrophoresis were electrophoretically transferred onto a nitrocellulose membrane, immunoblotted with polyclonal antibody against *R. rubrum* GlnB, and visualized with horseradish peroxidase color detection reagents (Bio-Rad, Richmond, CA).

## RESULTS

Different extraction methods yield very different measurements of ATP concentrations in *R. rubrum*. To monitor the ATP concentration in *R. rubrum* that was grown in MG medium, four extraction methods were used, as described in detail in Materials and Methods. As shown in Table 1, the TCA method gave the highest values, which is consistent with previous observations made with other bacteria (37, 46). Formic acid extraction gave slightly lower values. The boiling method is very simple, but this method and formaldehyde extraction gave much lower values than the other two methods for *R. rubrum*. Given the challenges noted above, it is tempting to favor methods that give higher ATP values, since these high values suggest both that metabolism has been rapidly terminated and that ATP was effectively extracted. However, the biologically important value for ATP is probably not the total, but rather the pool available to the particular biological process in question. Because of the difficulty in knowing this by any in vitro methods, we considered an in vivo assay to complement these data.

**Development of a luciferase reporter system to measure ATP in vivo in *R. rubrum*.** Alternative techniques to measure ATP based on firefly luciferase have been developed and used in bacterial and mammalian cells. Luciferase has been injected into mammalian cells to monitor ATP (5, 36), and a luciferase reporter system has also been used to monitor ATP in intact cells of *E. coli* and *R. capsulatus* (10, 14, 22, 60). The main challenge for this assay in bacterial systems is that cells typically must be treated with permeabilizing drugs such as polymyxin B to allow diffusion of luciferin into cells. Presumably, this also allows time for ATP metabolism and flux through the bacterial membrane, so this treatment almost certainly interferes with one's ability to rapidly monitor ATP pools with confidence.

As described in Materials and Methods, an expression vector for the luciferase variant was constructed. This variant

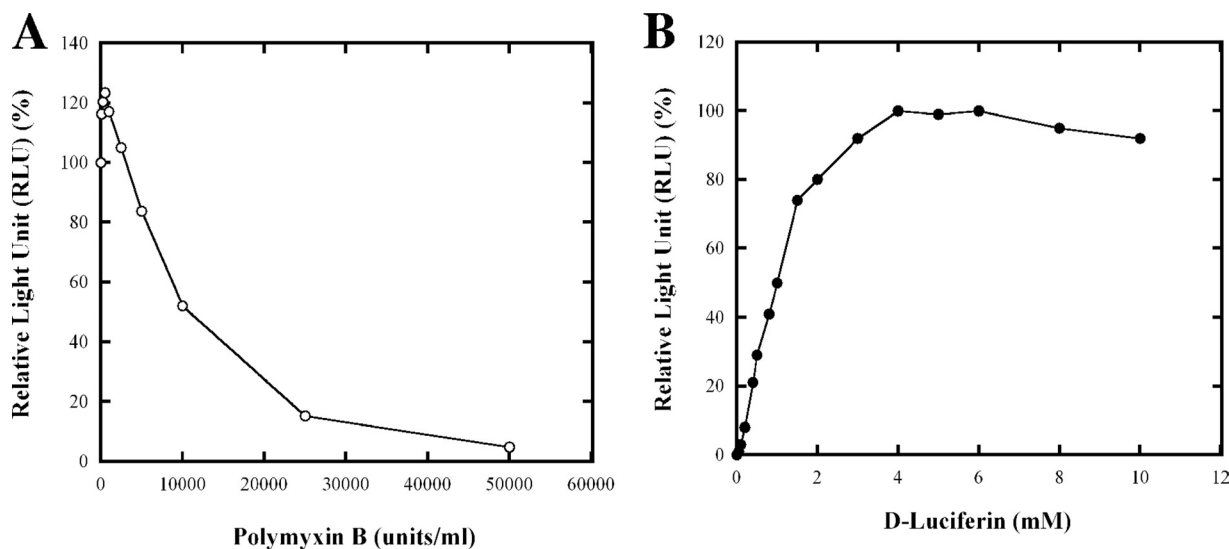


FIG. 1. In vivo analysis of ATP levels in *R. rubrum* strain UR2379 (wild type with plasmid pUX2524 carrying the luciferase reporter system). (A) Effect of the concentration of polymyxin B on the RLU at 4 mM luciferin. The values are normalized to the value obtained when polymyxin B concentration was at 0 mM. (B) Effect of the concentration of luciferin on the RLU in the absence of polymyxin B. The values are normalized to the maximum value obtained, when the luciferin concentration was 4 mM. Aliquots (10  $\mu$ l) of cells were mixed with 100  $\mu$ l of luciferase assay buffer solution, and the luminescence signal was measured with a luminometer for 2 min. Each point represents an average of at least three replicate assays, with a standard deviation of <10%.

(with a H245F substitution) has a  $K_m$  for Mg-ATP of 830  $\mu$ M, whereas the  $K_m$  for Mg-ATP of wild-type luciferase is 160  $\mu$ M (6), and is thus able to respond to the changes in ATP concentration in the predicted physiological range (14, 60). The gene encoding H245F luciferase expressed from the *aacCI* ( $Gm^r$  gene) promoter was cloned into a low-copy plasmid pRK404, yielding pUX2524. The otherwise wild-type *R. rubrum* strain with this plasmid was termed UR2379. We first titrated the RLU at different concentrations of polymyxin B and luciferin. As shown in Fig. 1A, in the presence of 4 mM luciferin, a high RLU was detected even without treatment of polymyxin B. Treatment with very low levels of polymyxin B caused a slight but reproducible increase in RLU, but concentrations >5,000 U/ml dramatically decreased the RLU. Thus, polymyxin B was not necessary for high luciferase detection in *R. rubrum*, which was surprising because of the completely opposite results seen in *R. capsulatus*, in which 10,000 to 50,000 U of polymyxin B/ml are required to reach the maximal RLU (14). As shown in Fig. 1B, in the absence of polymyxin B, the maximal RLU in *R. rubrum* was reached when the luciferin concentration in the assay was 4 to 6 mM. The apparent ability of luciferin to diffuse into the cells without treatment with polymyxin B dramatically simplifies the assay in *R. rubrum* and eliminates concerns over ATP loss. We then used this in vivo reporter system to monitor the change in ATP concentration in *R. rubrum* during dark-light shifts and  $NH_4^+$  addition.

**Treatment with uncoupling agents dramatically lowers the ATP pool and nitrogenase activity.** The uncoupling agents carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) have been previously used in *R. rubrum* to study the role of adenylate nucleotide pools in the regulation of nitrogenase activity (35, 52, 56). Both were shown to cause a rapid decrease of both the ATP pool and the nitrogenase activity. As a control for the

effectiveness of the in vivo luciferase reporter system in monitoring the changes in ATP pools, UR2379 was treated with FCCP or CCCP at final concentrations of 25 and 30  $\mu$ M, respectively, and the ATP concentration and nitrogenase activity were monitored in intact cells. As demonstrated in Fig. 2A, the ATP pool was rapidly diminished after the treatments, but some residual level of ATP remained. This result is consistent with previous results of ATP measurement after TCA extraction (52). Similarly, almost all nitrogenase activity was eliminated in 2 min after the treatment with both FCCP and CCCP (Fig. 2B). Thus, the luciferase method reports an extremely rapid and substantial drop in ATP levels, which correlate with a loss of nitrogenase activity.

**ATP concentration changes slightly in response to removal of light, but not after  $NH_4^+$  addition.** In *R. rubrum*, the DRAT/DRAG regulatory system controls nitrogenase activity in response to exogenous  $NH_4^+$  and to dark-light shifts (53, 80). One hypothesis for the regulation of DRAT/DRAG in response to a dark-light shifts has been that the shift to darkness causes a change in the ATP pool, which is sensed by  $P_{II}$ , and  $P_{II}$  in turn regulates DRAT/DRAG activities through protein-protein interactions. We therefore monitored changes of the ATP pool and nitrogenase activity in intact cells of UR2379 (wild-type *R. rubrum* expressing luciferase) during dark-light shifts and after  $NH_4^+$  addition. MG (malate-glutamate medium)-grown cultures were used for dark-light shifts, and  $MN^-$  (malate- $NH_4^+$ -free medium)-grown cultures were used for  $NH_4^+$  treatment, since these respond to  $NH_4^+$  more rapidly than do MG-grown cultures (77). As shown in Fig. 3A, the ATP concentration increased slightly but reproducibly just after the cells were shifted to dark and then decreased slowly. After 60 min of darkness, the ATP concentration was still ca. 90% of the initial level. ATP concentration increased ca. 10 to 15% after a return to light. The change in nitrogenase activity

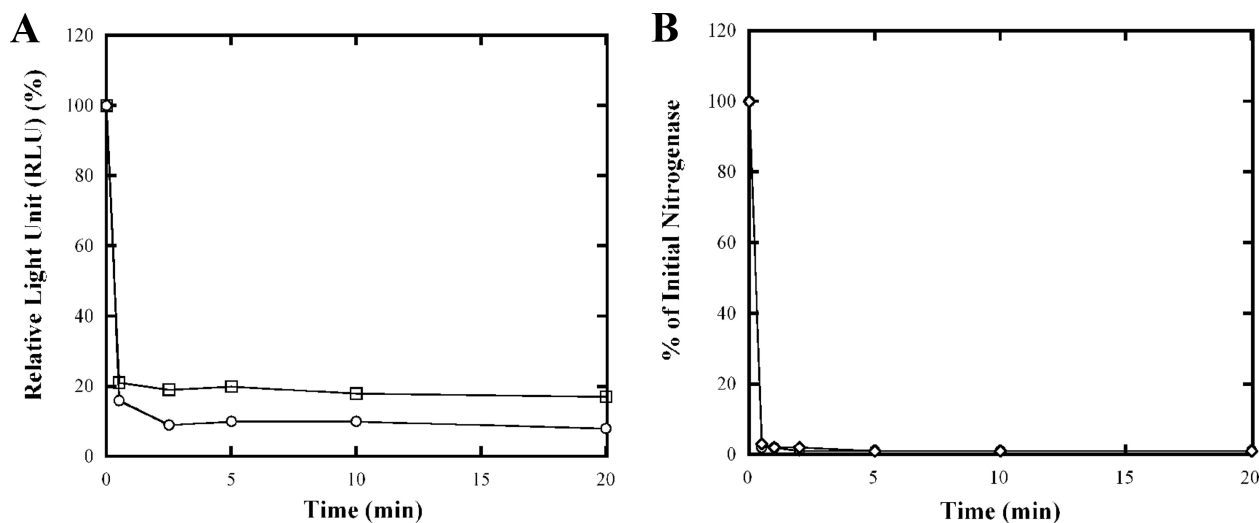


FIG. 2. Effect of FCCP and CCCP on ATP pools (A) and nitrogenase activity (B) in *R. rubrum* strain UR2379 (wild type carrying the luciferase reporter system). At time zero, FCCP (○) or CCCP (□) was added to MG-grown cultures at final concentrations of 25 and 30  $\mu\text{M}$ , respectively. At the times indicated, 1-ml aliquots of cells were withdrawn anaerobically and assayed under illumination for nitrogenase activity for 2 min. Initial nitrogenase activity (100%) in UR2379 was  $\sim 700$  nmol of ethylene produced per h per ml of cells at an  $\text{OD}_{600}$  of 1.0. Similarly, 10- $\mu\text{l}$  aliquots of cells were mixed with 100  $\mu\text{l}$  of luciferase assay buffer, and the luminescence signal was measured with a luminometer. The initial ATP concentration is  $\sim 85,000$  RLU/ml of cells at an  $\text{OD}_{600}$  of 1.0. Each point represents an average of at least three replicate assays, with standard deviation of  $<10\%$ .

was much more dramatic, as has been demonstrated numerous times (23, 41, 72).  $\text{NH}_4^+$  addition also had a dramatic effect on nitrogenase activity but an even less apparent effect on measurable ATP (Fig. 3B).

The very modest change in the ATP pool after a shift to dark was surprising to us, since we expected the change of the ATP pool would be more dramatic. It therefore seemed unlikely that the small change of the ATP pool in response

to these shifts is responsible for the dramatic regulation of nitrogenase activity.

**Construction of *R. rubrum purE* mutants to perturb ATP levels in vivo.** To further study the mechanism by which energy status affects nitrogenase regulation in *R. rubrum*, we constructed *purE* mutants, which are altered in the IMP synthesis pathway. In *E. coli*, IMP functions as a precursor for both AMP and GMP nucleotides, and the pathway consists of 11 enzymatic

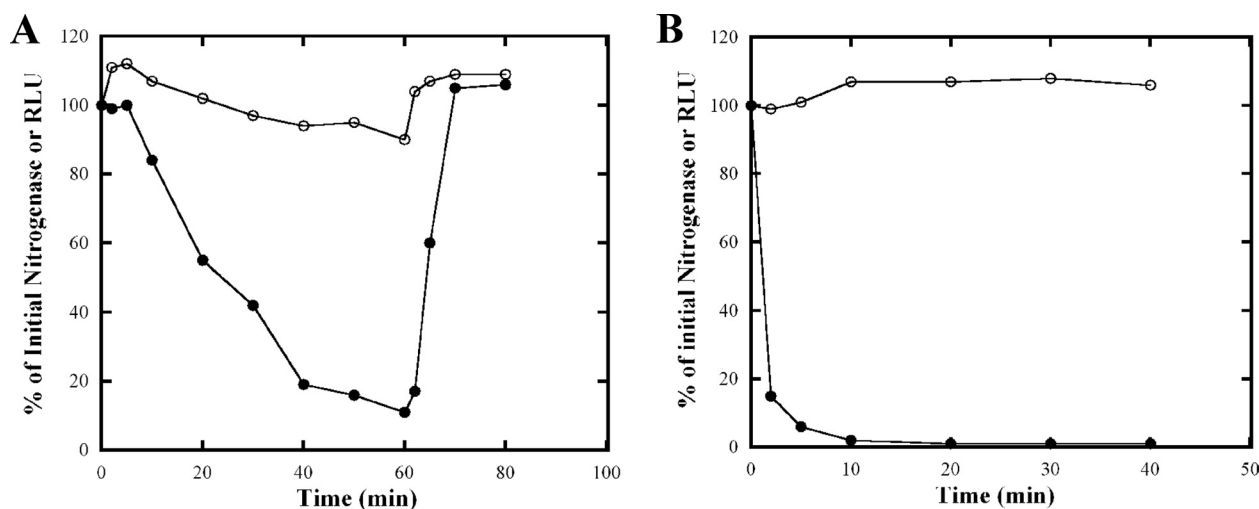


FIG. 3. Changes in nitrogenase activity and in vivo ATP concentration of *R. rubrum* UR2379 (wild type carrying the luciferase reporter system) in response to dark-light shifts (panel A) or the addition of  $\text{NH}_4^+$  (B). (A) UR2379 was grown in MG medium for about 2 days, and cells were shifted to dark at time zero and returned to light at 60 min. The nitrogenase activity (●) and ATP concentration (○) were measured as described in Fig. 2. Similar initial nitrogenase activity and ATP concentration were seen as in Fig. 2. (B) UR2379 was grown in  $\text{MN}^-$  medium for about 2 days, and  $\text{NH}_4\text{Cl}$  was added at final concentration of 10 mM at time zero. The nitrogenase activity (●) and ATP concentration (○) was measured as described in Fig. 2. The initial nitrogenase activity (100%) in UR2379 was  $\sim 200$  nmol of ethylene produce per h per ml of cells at an  $\text{OD}_{600}$  of 1.0. The initial ATP concentration was  $\sim 76,000$  RLU per ml of cells at an  $\text{OD}_{600}$  of 1.0.

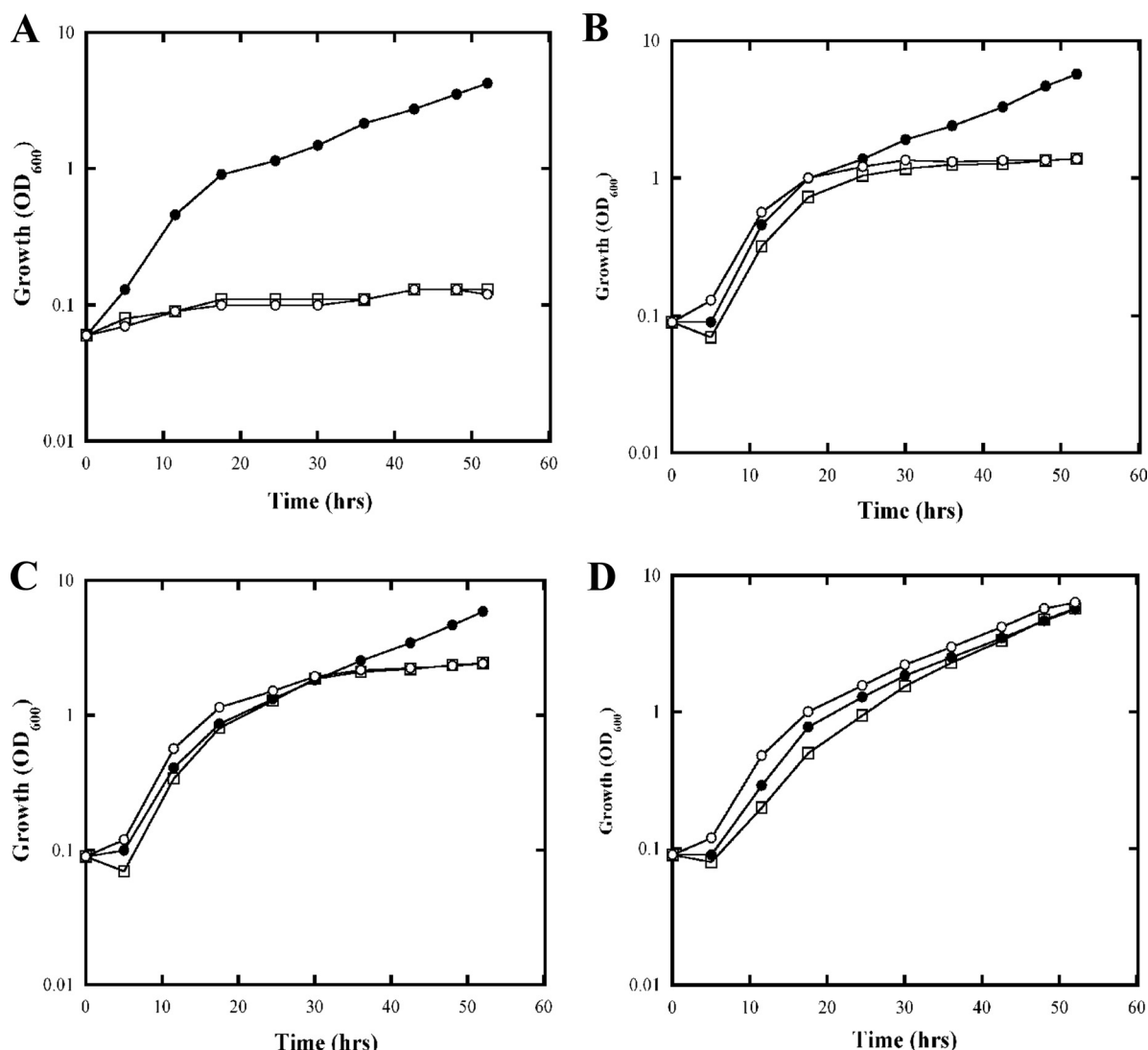


FIG. 4. Growth curve of UR2 (wild type) (●), UR2187 (*purE* mutant) (□), and UR2188 (*purE* mutant) (○) in MG with different concentrations of adenine: 0 mM (A), 0.05 mM (B), 0.1 mM (C), and 0.5 mM (D).

steps (75). In the sixth step 5'-phosphoribosylaminoimidazole (AIR) is converted to 5'-phosphoribosyl-*N*-5-aminoimidazole carboxylate (CAIR). Previously, this reaction was thought to be catalyzed by a single enzyme (AIR carboxylase), consisting of two subunits encoded by *purE* and *purK*. However, Mueller et al. established that these genes encode two different enzymes, *N*<sup>5</sup>-carboxyaminoimidazole ribonucleotide (NCAIR) isomerase/mutase and NCAIR synthetase, respectively (50).

As in *E. coli* and many other bacteria, *purE* and *purK* are genetically adjacent in *R. rubrum*. As described in Materials and Methods, *R. rubrum* mutants were constructed in which an *aacC1* gene was inserted into *purE* in the same orientation as *purE* (UR2187) or with the *aacC1* in the opposite orientation (UR2188).

These *purE* mutants are able to grow in rich (SMN) medium, but more slowly than wild type. They failed to grow in minimal MG medium, but they grew normally in SMN and MG media when supplemented with 0.5 mM adenine. To avoid selection

for suppressor mutations, we supplemented SMN medium with 0.5 mM adenine when we constructed these *purE* mutants.

The growth of *purE* mutants in MG medium was monitored at different levels of added adenine. As shown in Fig. 4A, *purE* mutants failed to grow in MG medium without adenine. When supplemented with 0.05 mM adenine in MG medium, *purE* mutants showed doubling times similar to those of the wild type initially but slowed down drastically when the OD<sub>600</sub> reached about 1 at about 24 h, presumably because the adenine was depleted (Fig. 4B). When supplemented with 0.1 mM adenine in MG medium, the growth of *purE* mutants slowed when the OD<sub>600</sub> reached about 2 at about 30 h (Fig. 4C), while 0.5 mM adenine allowed the *purE* mutants to grow similarly to the wild type (Fig. 4D). To study the effect of a perturbed level of ATP, we grew the *purE* mutant cultures in limiting adenine and examined the cultures after 40 to 44 h, where cultures were in either stationary phase (at 0.05 or 0.1 mM adenine) or late log phase (at 0.5 mM adenine).

TABLE 2. ATP levels in *R. rubrum* wild type and *purE* mutants when they were grown in MG medium containing 0.05, 0.1, or 0.5 mM adenine

Adenine concn (mM) and <i>R. rubrum</i> strain	Genotype and plasmid	Mean ATP level $\pm$ SD <sup>a</sup>	
		In vitro assay after TCA extraction (nmol/mg of dry wt)	In vivo luciferase assay (RLU/mg of dry wt)
0.05			
UR2	Wild type	4.46 $\pm$ 0.50	NT
UR2187	$\Delta$ <i>purE1::aacC1</i> mutant	1.04 $\pm$ 0.07	NT
UR2188	$\Delta$ <i>purE2::aacC1</i> mutant	0.89 $\pm$ 0.04	NT
UR2379	Wild type with pUX2524 (luciferase reporter)	3.93 $\pm$ 0.05	318,000 $\pm$ 12,500
UR2380	$\Delta$ <i>purE1::aacC1</i> mutant with pUX2524	1.07 $\pm$ 0.07	5,400 $\pm$ 1,000
UR2381	$\Delta$ <i>purE2::aacC1</i> mutant with pUX2524	1.04 $\pm$ 0.14	5,400 $\pm$ 900
0.1			
UR2	Wild type	4.39 $\pm$ 0.07	NT
UR2187	$\Delta$ <i>purE1::aacC1</i> mutant	1.04 $\pm$ 0.07	NT
UR2188	$\Delta$ <i>purE2::aacC1</i> mutant	0.96 $\pm$ 0.04	NT
UR2379	Wild type with pUX2524	5.18 $\pm$ 0.32	492,000 $\pm$ 33,900
UR2380	$\Delta$ <i>purE1::aacC1</i> mutant with pUX2524	1.14 $\pm$ 0.02	21,000 $\pm$ 1,300
UR2381	$\Delta$ <i>purE2::aacC1</i> mutant with pUX2524	1.21 $\pm$ 0.14	19,000 $\pm$ 2,300
0.5			
UR2	Wild type	4.39 $\pm$ 0.14	NT
UR2187	$\Delta$ <i>purE1::aacC1</i> mutant	2.78 $\pm$ 0.07	NT
UR2188	$\Delta$ <i>purE2::aacC1</i> mutant	3.14 $\pm$ 0.14	NT
UR2379	Wild type with pUX2524	4.50 $\pm$ 0.61	563,000 $\pm$ 34,600
UR2380	$\Delta$ <i>purE1::aacC1</i> mutant with pUX2524	3.25 $\pm$ 0.28	332,000 $\pm$ 30,000
UR2381	$\Delta$ <i>purE2::aacC1</i> mutant with pUX2524	3.46 $\pm$ 0.32	402,000 $\pm$ 49,600

<sup>a</sup> ATP levels were determined by in vitro assay after TCA extraction or determined by directly in vivo assay with luciferase reporter system as described in Materials and Methods. NT, not tested.

**The ATP pool in *R. rubrum purE* mutants is dramatically perturbed by limiting adenine.** pUX2524 (expressing H245F luciferase) was transferred into the *purE* mutants (UR2187 and UR2188), yielding UR2380 and UR2381. To study the effect of depletion of ATP on the nitrogenase activity, cells were grown in MG with different levels of adenine for 40 to 44 h to either late log or stationary phase as shown in Fig. 4. The ATP concentration was measured directly in vivo with the luciferase reporter system, as well as in vitro after TCA extraction. In this assay, the values for the wild type and the *purE* mutants cannot be compared with precision because the exact values for the mutants are a function of their degree of adenine starvation. Nevertheless, this allows us to compare methods and approximate the ATP levels in the wild type and the *purE* mutants.

As shown in Table 2, when assayed after TCA extraction, the ATP concentration in *purE* mutants (UR2187, UR2188, UR2380, and UR2381) is ca. 25% of that seen in wild-type controls (UR2 and UR2379) when grown in MG with 0.05 or 0.1 mM adenine. However, the ATP concentration in these *purE* mutants is much higher (ca. 70% of the wild type) when grown in MG with 0.5 mM adenine. When directly measured in intact cells, the ATP concentration in the *purE* mutants is less than 5% of that seen in wild-type controls when grown in MG with 0.05 or 0.1 mM adenine. As seen with the in vitro assay, the ATP concentration in these *purE* mutants was more comparable to that of the wild type when grown in MG with 0.5 mM adenine. Thus, both methods show that adenine-starved *purE* mutants have significantly lower ATP levels, although the magnitude of these differences is much greater using the lucif-

erase assay. We assume that the in vitro assay measures the total ATP pool and the in vivo luciferase system measures some free ATP pool, and it seems plausible that the latter pools would be more dramatically affected by adenine depletion.

**Regulation of nitrogenase activity in response to dark-light shifts in these *purE* mutants.** As mentioned in the introduction, because nitrogen fixation catalyzed by nitrogenase is a very energy-demanding process, the nitrogenase activity in *R. rubrum* is tightly regulated at both transcriptional and post-translational levels. First, the transcription of the *nif* operon is dependent on the NifA activity, which is regulated by GlnB. Second, nitrogenase activity is regulated posttranslationally by DRAT/DRAG. Under N<sub>2</sub>-fixing conditions, DRAG is active and DRAT is inactive, but after the addition of NH<sub>4</sub><sup>+</sup> excess or a shift to darkness, DRAG becomes inactive and DRAT becomes transiently active, resulting in the inactivation of dinitrogenase reductase. Both DRAT and DRAG eventually become inactive in the dark. When cultures are shifted back to the light, DRAG becomes active again and reactivates dinitrogenase reductase (80). The regulation of DRAT and DRAG activities certainly involves P<sub>II</sub> and therefore must also depend on pools of certain small molecules in the cell (25–27, 66, 70, 80).

An additional reason for expecting to see an effect of lower ATP levels on nitrogenase activity is because of the nature of the nitrogenase enzyme complex itself. Because of the concomitant and obligate reduction of protons to H<sub>2</sub>, the reduction of N<sub>2</sub> to NH<sub>3</sub> involves eight electron transfers between dinitrogenase and its terminal electron donor, dinitrogenase

TABLE 3. Nitrogenase activity and its response to dark-light shifts in *R. rubrum purE* mutants in MG with different concentrations of adenine

Adenine concn (mM) and <i>R. rubrum</i> strain	Chromosomal genotype	Nitrogenase activity		
		Initial level (nmol of ethylene/h/ml) <sup>a</sup>	% Activity <sup>b</sup>	
			40 min, dark	10 min, light
0				
UR2	Wild type	800	13	108
UR2187	<i>purE1::aacC1</i>	<20	NT	NT
UR2188	<i>purE2::aacC1</i>	<20	NT	NT
0.05				
UR2	Wild type	700	7	105
UR2187	<i>purE1::aacC1</i>	450	21	25
UR2188	<i>purE2::aacC1</i>	550	27	35
0.1				
UR2	Wild type	600	10	105
UR2187	<i>purE1::aacC1</i>	450	20	22
UR2188	<i>purE2::aacC1</i>	450	25	27
0.5				
UR2	Wild type	450	8	104
UR2187	<i>purE1::aacC1</i>	400	8	97
UR2188	<i>purE2::aacC1</i>	350	9	94

<sup>a</sup> The nitrogenase activity was derepressed in MG medium with different levels of adenine under illumination. Each unit of nitrogenase activity is expressed as the nmol of ethylene produced per h per ml of cells at an optical density at 600 nm of 1. Each activity value is from at least five replicate assays from different individually grown cultures. The standard deviation is about 10 to 15%. Initial levels were considered the 100% levels.

<sup>b</sup> That is, the percentage of the initial nitrogenase activity remaining after a shift to darkness or reillumination (light).

reductase (8). Each electron transfer requires the hydrolysis of two ATP molecules by dinitrogenase reductase, so one might suppose that nitrogenase activity would be sensitive to ATP levels even in the absence of the different levels of regulation.

Prior to the analysis of the ATP levels reported in Table 2, the same cultures were analyzed for nitrogenase activity. As shown in Table 3, little nitrogenase activity was seen in the *purE* mutants when grown in MG without adenine because of very poor growth. When supplemented with 0.05 or 0.1 mM adenine, the *purE* mutants showed moderate nitrogenase activity and responded normally to dark shift, but poor recovery of nitrogenase activity was seen after the cultures were returned back to light (Table 3). However, these *purE* mutants showed a normal recovery of nitrogenase activity when grown in MG medium supplemented with 0.5 mM adenine. The initial nitrogenase activity was somewhat lower in the wild type and the *purE* mutants when they were grown in the presence of 0.5 mM adenine, perhaps because of some indirect adenine inhibition of nitrogenase. This inhibition was seen in the wild type when treated with high levels of adenine (data not shown). Because the ATP assays in Table 2 were performed on the same cultures, we can conclude that limiting adenine caused a dramatic decrease in the ATP concentration, but remarkably little effect on nitrogenase activity. At most, the very low ATP pools do correlate with a poor recovery of nitrogenase activity by the DRAT/DRAG system after restoration of light.

Nitrogenase activity is a measure of the competing activities of DRAT and DRAG, so the failure of these cultures to re-

TABLE 4. Nitrogenase activity in *R. rubrum purE draG* mutants in MG with different concentrations of adenine

Adenine concn (mM) and <i>R. rubrum</i> strain	Chromosomal genotype	Nitrogenase activity (nmol of ethylene/h/ml)
0.05		
UR2	Wild type	750
UR2187	<i>purE1::aacC1</i>	420
UR2330	<i>purE1::aacC1 draG4::kan</i>	80
UR2378	<i>purE2::aacC1 draG4::kan</i>	70
0.1		
UR2	Wild type	650
UR2187	<i>purE1::aacC1</i>	500
UR2330	<i>purE1::aacC1 draG4::kan</i>	70
UR2378	<i>purE2::aacC1 draG4::kan</i>	70
0.5		
UR2	Wild type	500
UR2187	<i>purE1::aacC1</i>	400
UR2330	<i>purE1::aacC1 draG4::kan</i>	<20
UR2378	<i>purE2::aacC1 draG4::kan</i>	<20

cover nitrogenase activity after the restoration of light might indicate that the activity of either of these enzymes is altered. The use of a *draG* mutant has allowed us to study DRAT activity without the interference of DRAG activity (41), so we constructed *purE draG* double mutants to examine the impact of the ATP pool on DRAT activity alone. Adenine limitation caused substantial amounts of DRAT to be activated under derepression conditions, resulting in the low initial activity in *purE draG* mutants (Table 4). The low nitrogenase activity is caused by the modification of dinitrogenase reductase by DRAT, as confirmed by Western blots of the protein (data not shown). Thus, DRAT is inappropriately active under these conditions. Because of this result, the relatively high nitrogenase activity in the *DraG*<sup>+</sup> background indicates that DRAG is still active under these conditions. These results indicated that the adenine limitation in *purE* mutants significantly perturbs the regulation of both DRAT and DRAG activities, although these *purE* mutants showed moderate nitrogenase activity and appeared to respond to dark shifts normally.

**The modification of GlnB in response to dark-light shifts in wild type and *purE* mutants.** As mentioned in the Introduction, the DRAT/DRAG regulatory system is itself regulated by P<sub>II</sub>. We therefore monitored the modification of P<sub>II</sub> state in both the wild type and *purE* mutants in response to dark-light shifts to test if the low ATP pool in *purE* mutants has any effect on the P<sub>II</sub> modification. As shown in Fig. 5, in UR2 (wild type) most GlnB is accumulated in a UMP-modified form under derepression conditions (N-limiting conditions), and it becomes deuridylylated after a shift to darkness. When returned to the light, most GlnB becomes uridylylated again. The pattern of the modification of GlnB is consistent with the current model that P<sub>II</sub> activates DRAT and inactivates DRAG under nitrogen-excess conditions, while P<sub>II</sub>-UMP inhibits DRAT and activates DRAG under nitrogen-deficient conditions. The modification of GlnB is consistent with the regulation of nitrogenase and the modification of dinitrogenase activity. Similar to that seen in UR2, most GlnB in the *purE* mutant (UR2187) is accumulated in the modified form under dere-



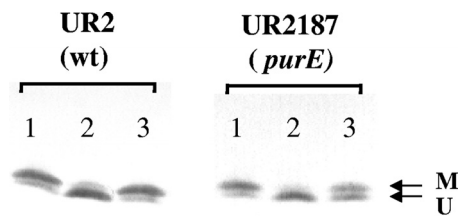


FIG. 5. Western immunoblots of GlnB in UR2 (wild type) and UR2187 (*purE* mutant). Cells were grown in MG medium with 0.1 mM adenine for about 2 days, with  $OD_{600}$  at 2.0 to 2.5. Then, 1-ml portions of the same culture were rapidly extracted by TCA precipitation of derepressed cultures of UR2 and UR2187 (lane 1), 60 min after a shift to dark (lane 2), and 10 min after a return to light (lane 3). Protein samples were loaded on low cross-linker SDS-Tricine gels and immunoblotted with antibody to *R. rubrum* GlnB. Arrow M indicates the position of the modified subunit, and arrow U indicates the position of the unmodified subunit.

pression conditions and it becomes deuridylylated after a shift to darkness. However, only a small part of GlnB becomes modified again after a return to light, which is also consistent with the failure of this mutant to recover nitrogenase activity (Table 3). Similar to the modification of GlnB, the modification of GlnJ was also altered; a poor reuridylylation of GlnJ was also seen in *purE* mutants after a return to light (data not shown). These results indicate that adenine depletion has significant effects on  $P_{II}$  modification, especially on uridylylation after restoration of light. The failure of uridylylation of  $P_{II}$  presumably affects its role in the reactivation of DRAG and the restoration of nitrogenase activity after a return to light.

## DISCUSSION

In some nitrogen-fixing bacteria, such as *R. rubrum*, *A. brasilense* and *R. capsulatus*, nitrogenase activity is regulated not only by the addition of  $NH_4^+$  but also in response to energy depletion (by a dark shift for *R. rubrum* and *R. capsulatus* or an anaerobic shift for *A. brasilense*) (35, 48, 74, 80). Although the change of ATP levels might be the basis for these effects, previous measurements of the ATP pool yielded inconsistent results. Li et al. reported that the pool of ATP in *R. rubrum* decreased after the dark shift, stayed at low levels, and then recovered after cells were returned to light (40). However, using a different extraction method, Paul and Ludden saw the pool of ATP increase rapidly after the dark shift and then decrease slightly, but they also reported further ATP fluctuations during dark incubation (56). In response to  $NH_4^+$  addition, no obvious changes in ATP levels were detected in *R. rubrum* and *K. pneumoniae*, although a change was seen with *Clostridium pasteurianum* (56, 68). However, the ADP/ATP ratio decreased in the latter two organisms when they were switched from  $N_2$  to  $NH_4^+$  as the sole N source (68).

Given our data and the concerns about the analysis of ATP levels noted in the Introduction, it is difficult to know how to interpret the above results. There seems to be no doubt that detected ATP levels are highly dependent on the extraction method used. We therefore favor the luciferase assay, since it minimally perturbs the cells and reports at least some aspect of "available" ATP. This assay also displayed a particular advan-

tage when used with *R. rubrum*: surprisingly, luciferin appears to be able to rapidly penetrate into *R. rubrum* cells without any treatment. This is in contrast to the long treatment time (>30 min) with a low concentration of polymyxin B as originally used for *E. coli* (10), and a shortened treatment at high polymyxin B concentration used subsequently (60). Thus, without the treatment of polymyxin B we are able to monitor rapid changes in the ATP pool in *R. rubrum* without any potential ATP loss or delay.

When applied to *R. rubrum*, this method suggests very modest changes in ATP after removal of light and essentially no change after the addition of  $NH_4^+$ . At the very least, this result implies that ATP levels per se are not the critical signal for the dramatic posttranslational regulation of nitrogenase that we monitor.

Our use of *purE* mutants showed that we could severely perturb ATP levels in vivo, as determined by both luciferase and an extraction method. Indeed, given the striking change in detected ATP pools, it is very surprising to us that moderate nitrogenase activity was seen in *purE* mutants under these adenine-limiting conditions (ca. 70% of that seen in wild type). This result implies that relatively low levels of ATP are sufficient to maintain a fairly robust flux of electrons through nitrogenase itself, and DRAG is highly active and keeps dinitrogenase reductase in its active form. Indeed, we confirmed the latter point with the use of the *purE draG* strain. The only apparent flaw in the regulation in these *purE* mutants under adenine-depletion conditions was their poor recovery of nitrogenase activity after a return to light. A direct analysis of the modification state of  $P_{II}$  showed that a substantial fraction of the  $P_{II}$  in *purE* mutants remained in the deuridylylated form after the return to light, whereas  $P_{II}$  in the wild type became uridylylated under the same conditions. This indicates that adenine depletion and low ATP levels have significant effects on  $P_{II}$  modification, which in turn alters the DRAT/DRAG regulatory system.

The current hypothesis is that both DRAT and DRAG activities are inhibited in vivo by binding different forms of  $P_{II}$  proteins. DRAT- $P_{II}$  interaction was observed with yeast two-hybrid system in *R. capsulatus* and *R. rubrum* (57, 83). Pull-down experiments with *A. brasilense* strains overexpressing His-tagged DRAT or DRAG indicated that DRAT binds only unmodified  $P_{II}$  and DRAG binds both unmodified and modified  $P_{II}$  (25), although recent in vitro studies showed that DRAT can also bind modified  $P_{II}$  under some conditions (26). DRAG- $P_{II}$  interaction was observed in vitro as well, although no interaction was seen in the yeast two-hybrid system.

So how might we imagine a role for the relative levels of ADP and ATP in mitigating these activities, even without the change of  $P_{II}$  modification state? The formation of DRAG- $P_{II}$  complex is stimulated by ADP, and partially dissociated by either  $\alpha$ -KG or ATP (27). The DRAG- $P_{II}$  complex can also interact with membrane protein AmtB, and the membrane sequestration removes DRAG from its substrate, dinitrogenase reductase. The interaction of *R. rubrum* GlnJ and AmtB<sub>1</sub> is also affected by  $\alpha$ -KG, ADP, and ATP (71). Thus, competition between ADP and ATP for  $P_{II}$  could affect the availability of DRAG to support nitrogenase activity. The case for ADP/ATP effects on DRAT is less clear, but the simple notion is that ADP-bound, but not ATP-bound  $P_{II}$  would bind DRAT

to stimulate its activity. Under energy excess conditions, where ADP might be low, P<sub>II</sub> is saturated by ATP and  $\alpha$ -KG and is uridylylated by GlnD, and this would lead it to dissociate from DRAT, resulting in the loss of DRAT activity. In the presence of  $\alpha$ -KG, either ADP or ATP can support modification of P<sub>II</sub>, but the behavior of modified P<sub>II</sub> with ATP- and ADP-bound P<sub>II</sub> might well be different in terms of interaction with DRAT. Thus, under energy-limiting conditions, P<sub>II</sub> is likely in the ADP-bound form (modified or not), and it could activate DRAT. We emphasize that these rationalizations are highly speculative and are merely a framework to consider possible ADP/ATP effects.

Although not related to energy-sensing by P<sub>II</sub>, we also note that a severe lowering of ATP levels has little impact on the electron flux through dinitrogenase. This surprises us because each electron transfer to dinitrogenase requires hydrolysis of two molecules of ATP and might have been expected to be very sensitive to ATP levels. We have no data to address this result, but it is our working hypothesis that the affinity of dinitrogenase reductase for ATP is sufficiently high, so that it competes particularly well for ATP. This result has implications for efforts to enhance H<sub>2</sub> production through nitrogenase for bioenergy purposes.

What role does ATP have in modulating P<sub>II</sub> function in vivo? The simple answer is that ATP cannot be the sole signal, and we suppose that the ratio of ADP/ATP might be the key issue. Although the impact of ATP or the ADP/ATP ratio has not previously been analyzed in vivo, the notion of the ADP/ATP ratio has been supported by a variety of in vitro analyses. Our previous results showed that the interaction of *R. rubrum* GlnJ and AmtB<sub>1</sub> is disrupted by  $\alpha$ -KG and/or ATP, while ADP inhibits the destabilization of the GlnJ-AmtB<sub>1</sub> complex in the presence of ATP and  $\alpha$ -KG. These results support a role for P<sub>II</sub> as an energy sensor that measures the ratio of ATP to ADP (71). Recently, several lines of in vitro evidence suggest that the ratio of ADP/ATP plays an important role in the interaction of P<sub>II</sub> with its different receptors in different bacteria. Jiang and Ninfa have shown that ADP has a significant effect on P<sub>II</sub> function in the regulation of NtrB and GlnE in an in vitro *E. coli* system, and their results indicate that P<sub>II</sub> acts as a sensor of both ATP and ADP signals (31). Teixeira et al. found that the interaction of *R. rubrum* GlnJ with its targets (AmtB<sub>1</sub>, GlnD, and GlnE) is also dependent on the ratio of ADP/ATP in vitro and that the ADP/ATP ratio can override the  $\alpha$ -KG signal (66). Finally, Huergo et al. reported that ATP, ADP, and  $\alpha$ -KG play a key role in *A. brasilense* P<sub>II</sub> interaction with the DRAT/DRAG regulatory protein in vitro (26). Consistent with an important role for ADP binding by P<sub>II</sub>, the crystal structures of *Thermotoga maritima* P<sub>II</sub> and the *E. coli* P<sub>II</sub>-AmtB protein complex show ADP bound to P<sub>II</sub> even though no ADP was added during crystallization (9, 61).

Unfortunately, we are not in a position to address the level of ADP in the cell under these conditions. The luciferase assay is not useful because it only reports the presence of ATP. Although some extraction methods for ADP have been described, these have technical challenges similar to those for ATP quantitation, with different extraction methods yielding dramatically different ADP values (37, 46). The TCA extraction method is the most effective for extraction of both ATP and ADP. However, the TCA has to be removed by an addi-

tional extraction method so the ADP/ATP can be enzymatically converted to ATP. These multiple-step manipulations greatly reduce the reliability of ADP quantitation. Thus, we are skeptical that values from these analyses are informative.

In conclusion, the data show that removal and restoration of light to cultures of *R. rubrum* has dramatic effects on nitrogenase activity and the modification status of P<sub>II</sub>, but the plausible hypothesis that these effects reflect a change in ATP levels seems to be incorrect. It is therefore our working hypothesis, based primarily on the in vitro analyses noted above, that either ADP levels or the ADP/ATP ratio is the primary metabolic signal, although a completely new method of analysis will be necessary to test that hypothesis. We also saw that a dramatic lowering of ATP in the *purE* mutants had little impact on nitrogenase activity, suggesting that this system is surprisingly effective in competing with other ATP-utilizing systems in the cell.

#### ACKNOWLEDGMENTS

This study was supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, and NIGMS grant GM65891 to G.P.R.

We thank Richard Gourse and Tamas Gaal for generously providing the *luc* plasmid and the TD-20/20 luminometer for the luciferase assay, Mary Conrad and Jose Serate for the purification of GlnB, and David Wolfe and Emily Welch for valuable criticisms of the manuscript.

#### REFERENCES

1. Arcondéguy, T., R. Jack, and M. Merrick. 2001. P<sub>II</sub> signal transduction proteins, pivotal players in microbial nitrogen control. *Microbiol. Mol. Biol. Rev.* **65**:80–105.
2. Arsene, F., P. A. Kaminski, and C. Elmerich. 1996. Modulation of NifA activity by P<sub>II</sub> in *Azospirillum brasilense*: evidence for a regulatory role of the NifA N-terminal domain. *J. Bacteriol.* **178**:4830–4838.
3. Atlante, A., S. Giannattasio, A. Bobba, S. Gagliardi, V. Petragallo, P. Calissano, E. Marra, and S. Passarella. 2005. An increase in the ATP levels occurs in cerebellar granule cells en route to apoptosis in which ATP derives from both oxidative phosphorylation and anaerobic glycolysis. *Biochim. Biophys. Acta* **1708**:50–62.
4. Benelli, E. M., E. M. Souza, S. Funayama, L. U. Rigo, and F. O. Pedrosa. 1997. Evidence for two possible *glnB*-type genes in *Herbaspirillum seropedicae*. *J. Bacteriol.* **179**:4623–4626.
5. Bowers, K. C., A. P. Allshire, and P. H. Cobbold. 1992. Bioluminescent measurement in single cardiomyocytes of sudden cytosolic ATP depletion coincident with rigor. *J. Mol. Cell Cardiol.* **24**:213–218.
6. Branchini, B. R., R. A. Magyar, M. H. Murtiashaw, S. M. Anderson, and M. Zimmer. 1998. Site-directed mutagenesis of histidine 245 in firefly luciferase: a proposed model of the active site. *Biochemistry* **37**:15311–15319.
7. Burgener, A., K. Coombs, and M. Butler. 2006. Intracellular ATP and total adenylate concentrations are critical predictors of reovirus productivity from Vero cells. *Biotechnol. Bioeng.* **94**:667–679.
8. Burris, R. H. 1991. Nitrogenases. *J. Biol. Chem.* **266**:9339–9342.
9. Conroy, M. J., A. Durand, D. Lupo, X. D. Li, P. A. Bullough, F. K. Winkler, and M. Merrick. 2007. The crystal structure of the *Escherichia coli* AmtB-GlnK complex reveals how GlnK regulates the ammonia channel. *Proc. Natl. Acad. Sci. USA* **104**:1213–1218.
10. Dement'eva, E. I., N. N. Ugarova, and P. H. Cobbold. 1996. Measurement of ATP in intact *Escherichia coli* cells, containing recombinant firefly luciferase. *Biokhimiia* **61**:1285–1293.
11. Dennis, P. B., A. Jaeschke, M. Saitoh, B. Fowler, S. C. Kozma, and G. Thomas. 2001. Mammalian TOR: a homeostatic ATP sensor. *Science* **294**:1102–1105.
12. de Zamaroczy, M., A. Paquelin, G. Peltre, K. Forchhammer, and C. Elmerich. 1996. Coexistence of two structurally similar but functionally different P<sub>II</sub> proteins in *Azospirillum brasilense*. *J. Bacteriol.* **178**:4143–4149.
13. Dhar-Chowdhury, P., B. Malester, P. Rajacic, and W. A. Coetzee. 2007. The regulation of ion channels and transporters by glycolytically derived ATP. *Cell. Mol. Life Sci.* **64**:3069–3083.
14. Di Tomaso, G., R. Borghese, and D. Zannoni. 2001. Assay of ATP in intact cells of the facultative phototroph *Rhodobacter capsulatus* expressing recombinant firefly luciferase. *Arch. Microbiol.* **177**:11–19.
15. Ditta, G., T. Schmidhauser, E. Yakobson, P. Lu, X.-W. Liang, D. R. Finlay, D. Guiney, and D. R. Helinski. 1985. Plasmids related to the broad host

- range vector, pRK290, coding for gene cloning and for monitoring gene expression. *Plasmid* **13**:149–153.
16. **Dodsworth, J. A., N. C. Cady, and J. A. Leigh.** 2005. 2-Oxoglutarate and the PII homologues NifI, and NifL regulate nitrogenase activity in cell extracts of *Methanococcus marisplacidus*. *Mol. Microbiol.* **56**:1527–1538.
  17. **Dodsworth, J. A., and J. A. Leigh.** 2006. Regulation of nitrogenase by 2-oxoglutarate-reversible, direct binding of a PII-like nitrogen sensor protein to dinitrogenase. *Proc. Natl. Acad. Sci. USA* **103**:9779–9784.
  18. **Drepper, T., S. Gross, A. F. Yakunin, P. C. Hallenbeck, B. Masepohl, and W. Klipp.** 2003. Role of GlnB and GlnK in ammonium control of both nitrogenase systems in the phototrophic bacterium *Rhodobacter capsulatus*. *Microbiology* **149**:2203–2212.
  19. **Fitzmaurice, W. P., L. L. Saari, R. G. Lowery, P. W. Ludden, and G. P. Roberts.** 1989. Genes coding for the reversible ADP-ribosylation system of dinitrogenase reductase from *Rhodospirillum rubrum*. *Mol. Gen. Genet.* **218**:340–347.
  20. **Forchhammer, K.** 2008. P<sub>II</sub> signal transducers: novel functional and structural insights. *Trends Microbiol.* **16**:65–72.
  21. **Fu, H. A., A. Hartmann, R. G. Lowery, W. P. Fitzmaurice, G. P. Roberts, and R. H. Burris.** 1989. Posttranslational regulatory system for nitrogenase activity in *Azospirillum* spp. *J. Bacteriol.* **171**:4679–4685.
  22. **Funabashi, H., T. Imajo, J. Kojima, E. Kobatake, and M. Aizawa.** 1999. Bioluminescent monitoring of intracellular ATP during fermentation. *Luminescence* **14**:291–296.
  23. **Grunwald, S. K., D. P. Lies, G. P. Roberts, and P. W. Ludden.** 1995. Post-translational regulation of nitrogenase in *Rhodospirillum rubrum* strains overexpressing the regulatory enzymes dinitrogenase reductase ADP-ribosyltransferase and dinitrogenase reductase activating glycohydrolase. *J. Bacteriol.* **177**:628–635.
  24. **He, L., E. Soupene, A. Ninfa, and S. Kustu.** 1998. Physiological role for the GlnK protein of enteric bacteria: relief of NifL inhibition under nitrogen-limiting conditions. *J. Bacteriol.* **180**:6661–6667.
  25. **Huergo, L. F., L. S. Chubatsu, E. M. Souza, F. O. Pedrosa, M. B. R. Steffens, and M. Merrick.** 2006. Interactions between P<sub>II</sub> proteins and the nitrogenase regulatory enzymes DraT and DraG in *Azospirillum brasilense*. *FEBS Lett.* **580**:5232–5236.
  26. **Huergo, L. F., M. Merrick, R. A. Monteiro, L. S. Chubatsu, M. B. R. Steffens, F. O. Pedrosa, and E. M. Souza.** 2009. In vitro interactions between the P<sub>II</sub> proteins and the nitrogenase regulatory enzymes dinitrogenase reductase ADP-ribosyltransferase (DraT) and dinitrogenase reductase-activating glycohydrolase (DraG) in *Azospirillum brasilense*. *J. Biol. Chem.* **284**:6674–6682.
  27. **Huergo, L. F., M. Merrick, F. O. Pedrosa, L. S. Chubatsu, M. S. Araujo, and E. M. Souza.** 2006. Ternary complex formation between AmtB, GlnZ and the nitrogenase regulatory enzyme DraG reveals a novel facet of nitrogen regulation in bacteria. *Mol. Microbiol.* **66**:1523–1535.
  28. **Huergo, L. F., E. M. Souza, M. S. Araujo, F. O. Pedrosa, L. S. Chubatsu, M. B. R. Steffens, and M. Merrick.** 2006. ADP-ribosylation of dinitrogenase reductase in *Azospirillum brasilense* is regulated by AmtB-dependent membrane sequestration of DraG. *Mol. Microbiol.* **59**:326–337.
  29. **Jack, R., M. De Zamaroczy, and M. Merrick.** 1999. The signal transduction protein GlnK is required for NifL-dependent nitrogen control of *nif* gene expression in *Klebsiella pneumoniae*. *J. Bacteriol.* **181**:1156–1162.
  30. **Jensen, K. F., U. Houlberg, and P. Nygaard.** 1979. Thin-layer chromatographic methods to isolate <sup>32</sup>P-labeled 5-phosphoribosyl- $\alpha$ -1-pyrophosphate (PRPP): determination of cellular PRPP pools and assay of PRPP synthetase activity. *Anal. Biochem.* **98**:254–263.
  31. **Jiang, P., and A. J. Ninfa.** 2007. *Escherichia coli* PII signal transduction protein controlling nitrogen assimilation acts as a sensor of adenylate energy charge in vitro. *Biochemistry* **46**:12979–12996.
  32. **Jiang, P., J. A. Peliska, and A. J. Ninfa.** 1998. Enzymological characterization of the signal-transducing uridylyltransferase/uridylyl-removing enzyme (EC 2.7.7.59) of *Escherichia coli* and its interaction with the PII protein. *Biochemistry* **37**:12782–12794.
  33. **Jonsson, A., and S. Nordlund.** 2007. In vitro studies of the uridylylation of the three P<sub>II</sub> protein paralogs from *Rhodospirillum rubrum*: the transferase activity of *R. rubrum* GlnD is regulated by  $\alpha$ -ketoglutarate and divalent cations but not by glutamine. *J. Bacteriol.* **189**:3471–3478.
  34. **Kamberov, E. S., M. R. Atkinson, and A. J. Ninfa.** 1995. The *Escherichia coli* PII signal transduction protein is activated upon binding 2-ketoglutarate and ATP. *J. Biol. Chem.* **270**:17797–17807.
  35. **Kanemoto, R. H., and P. W. Ludden.** 1984. Effect of ammonia, darkness, and phenazine methosulfate on whole-cell nitrogenase activity and Fe protein modification in *Rhodospirillum rubrum*. *J. Bacteriol.* **158**:713–720.
  36. **Koop, A., and P. H. Cobbold.** 1993. Continuous bioluminescent monitoring of cytoplasmic ATP in single isolated rat hepatocytes during metabolic poisoning. *Biochem. J.* **295**:165–170.
  37. **Larsson, C.-M., and T. Olsson.** 1979. Firefly assay of adenine nucleotides from algae: comparison of extraction methods. *Plant Cell Physiol.* **20**:145–155.
  38. **Lehman, L. J., and G. P. Roberts.** 1991. Identification of an alternative nitrogenase system in *Rhodospirillum rubrum*. *J. Bacteriol.* **173**:5705–5711.
  39. **Leigh, J. A., and J. A. Dodsworth.** 2007. Nitrogen regulation in bacteria and archaea. *Annu. Rev. Microbiol.* **61**:349–377.
  40. **Li, J. D., C. Z. Hu, and D. C. Yoch.** 1987. Changes in amino acid and nucleotide pools of *Rhodospirillum rubrum* during switch-off of nitrogenase activity initiated by NH<sub>4</sub><sup>+</sup> or darkness. *J. Bacteriol.* **169**:231–237.
  41. **Liang, J. H., G. M. Nielsen, D. P. Lies, R. H. Burris, G. P. Roberts, and P. W. Ludden.** 1991. Mutations in the *draT* and *draG* genes of *Rhodospirillum rubrum* result in loss of regulation of nitrogenase by reversible ADP-ribosylation. *J. Bacteriol.* **173**:6903–6909.
  42. **Lies, D. P.** 1994. Genetic manipulation and the overexpression analysis of posttranslational nitrogen fixation regulation in *Rhodospirillum rubrum*. Ph.D. thesis. University of Wisconsin-Madison, Madison.
  43. **Little, R., and H. Bremer.** 1982. Quantitation of guanosine 5',3'-bisdiphosphate in extracts from bacterial cells by ion-pair reverse-phase high-performance liquid chromatography. *Anal. Biochem.* **126**:381–388.
  44. **Little, R., V. Colombo, A. Leech, and R. Dixon.** 2002. Direct interaction of the NifL regulatory protein with the GlnK signal transducer enables the *Azotobacter vinelandii* NifL-NifA regulatory system to respond to conditions replete for nitrogen. *J. Biol. Chem.* **277**:15472–15481.
  45. **Little, R., F. Reyes-Ramirez, Y. Zhang, W. C. van Heeswijk, and R. Dixon.** 2000. Signal transduction to the *Azotobacter vinelandii* NIFL-NIFA regulatory system is influenced directly by interaction with 2-oxoglutarate and the PII regulatory protein. *EMBO J.* **19**:6041–6050.
  46. **Lundin, A., and A. Thore.** 1975. Comparison of methods for extraction of bacterial adenine nucleotides determined by firefly assay. *Appl. Microbiol.* **30**:713–721.
  47. **Martin, D. E., T. Hurek, and B. Reinhold-Hurek.** 2000. Occurrence of three P<sub>II</sub>-like signal transmitter proteins in the diazotrophic proteobacterium *Azorarcus* sp. BH72. *Mol. Microbiol.* **38**:276–288.
  48. **Masepohl, B., T. Drepper, A. Paschen, S. Gross, A. Pawlowski, K. Raabe, K. U. Riedel, and W. Klipp.** 2002. Regulation of nitrogen fixation in the phototrophic purple bacterium *Rhodobacter capsulatus*. *J. Mol. Microbiol. Biotechnol.* **4**:243–248.
  49. **Masepohl, B., R. Krey, and W. Klipp.** 1993. The *draTG* gene region of *Rhodobacter capsulatus* is required for posttranslational regulation of both the molybdenum and the alternative nitrogenase. *J. Gen. Microbiol.* **139**:2667–2675.
  50. **Mueller, E. J., E. Meyer, J. Rudolph, V. J. Davisson, and J. Stubbe.** 1994. N<sup>5</sup>-carboxyaminoimidazole ribonucleotide: evidence for a new intermediate and two new enzymatic activities in the de novo purine biosynthetic pathway of *Escherichia coli*. *Biochemistry* **33**:2269–2278.
  51. **Ninfa, A. J., and M. R. Atkinson.** 2000. PII signal transduction proteins. *Trends Microbiol.* **8**:172–179.
  52. **Nordlund, S., and L. Höglund.** 1986. Studies of the adenylate and pyridine nucleotide pools during nitrogenase 'switch-off' in *Rhodospirillum rubrum*. *Plant Soil.* **90**:203–209.
  53. **Nordlund, S., and P. W. Ludden.** 2004. Post-translational regulation of nitrogenase in photosynthetic bacteria, p. 175–196. In W. Klipp, B. Masepohl, J. R. Gallon, and W. E. Newton (ed.), *Genetics and regulation of nitrogen fixation in free-living bacteria*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
  54. **Ohmori, M., and A. Hattori.** 1978. Transient change in the ATP pool of *Anabaena cylindrica* associated with ammonia assimilation. *Arch. Microbiol.* **117**:17–20.
  55. **Palmfeldt, J., M. Paese, B. Hahn-Hagerdal, and E. W. Van Niel.** 2004. The pool of ADP and ATP regulates anaerobic product formation in resting cells of *Lactococcus lactis*. *Appl. Environ. Microbiol.* **70**:5477–5484.
  56. **Paul, T. D., and P. W. Ludden.** 1984. Adenine nucleotide levels in *Rhodospirillum rubrum* during switch-off of whole-cell nitrogenase activity. *Biochem. J.* **224**:961–969.
  57. **Pawlowski, A., K. U. Riedel, W. Klipp, P. Dreiskemper, S. Groß, H. Bierhoff, T. Drepper, and B. Masepohl.** 2003. Yeast two-hybrid studies on interaction of proteins involved in regulation of nitrogen fixation in the phototrophic bacterium *Rhodobacter capsulatus*. *J. Bacteriol.* **185**:5240–5247.
  58. **Schägger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**:368–379.
  59. **Schneider, D. A., T. Gaal, and R. L. Gourse.** 2002. NTP-sensing by rRNA promoters in *Escherichia coli* is direct. *Proc. Natl. Acad. Sci. USA* **99**:8602–8607.
  60. **Schneider, D. A., and R. L. Gourse.** 2004. Relationship between growth rate and ATP concentration in *Escherichia coli*: a bioassay for available cellular ATP. *J. Biol. Chem.* **279**:8262–8268.
  61. **Schwarzenbacher, R., F. von Delft, P. Abdubek, E. Ambing, T. Biorac, L. S. Brinen, J. M. Canaves, J. Cambell, H. J. Chiu, X. Dai, et al.** 2004. Crystal structure of a putative PII-like signaling protein (TM0021) from *Thermotoga maritima* at 2.5 Å resolution. *Proteins* **54**:810–813.
  62. **Schweizer, H. P.** 1993. Small broad-host-range gentamycin resistance gene cassettes for site-specific insertion and deletion mutagenesis. *BioTechniques* **15**:831–834.
  63. **Shapiro, B. M., and E. R. Stadtman.** 1968. Glutamine synthetase deadenylating enzyme. *Biochem. Biophys. Res. Commun.* **30**:32–37.
  64. **Simon, R., U. B. Prierer, and A. Pühler.** 1983. A broad host range mobili-

- zation system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. *Bio/Technology* **1**:784–791.
65. **Stadtman, E. R.** 2001. The story of glutamine synthetase regulation. *J. Biol. Chem.* **276**:44357–44364.
66. **Teixeira, P. F., A. Jonsson, M. Frank, H. Wang, and S. Nordlund.** 2008. Interaction of the signal transduction protein GlnJ with the cellular targets AmtB1, GlnE, and GlnD in *Rhodospirillum rubrum*: dependence on manganese, 2-oxoglutarate, and the ADP/ATP ratio. *Microbiology* **154**:2336–2347.
67. **Tremblay, P. L., T. Drepper, B. Masepohl, and P. C. Hallenbeck.** 2007. Membrane sequestration of P<sub>II</sub> proteins and nitrogenase regulation in the photosynthetic bacterium *Rhodobacter capsulatus*. *J. Bacteriol.* **189**:5850–5859.
68. **Upchurch, R. G., and L. E. Mortenson.** 1980. In vivo energetics and control of nitrogen fixation: changes in the adenylate energy charge and adenosine 5'-diphosphate/adenosine 5'-triphosphate ratio of cells during growth on dinitrogen versus growth on ammonia. *J. Bacteriol.* **143**:274–284.
69. **van Heeswijk, W. C., S. Hoving, D. Molenaar, B. Stegeman, D. Kahn, and H. V. Westerhoff.** 1996. An alternative P<sub>II</sub> protein in the regulation of glutamine synthetase in *Escherichia coli*. *Mol. Microbiol.* **21**:133–146.
70. **Wang, H., C. C. Franke, S. Nordlund, and A. Norén.** 2005. Reversible membrane association of dinitrogenase reductase activating glycohydrolase in the regulation of nitrogenase activity in *Rhodospirillum rubrum*; dependence on GlnJ and AmtB1. *FEMS Microbiol. Lett.* **253**:273–279.
71. **Wolfe, D. M., Y. Zhang, and G. P. Roberts.** 2007. Specificity and regulation of interaction between the P<sub>II</sub> and AmtB<sub>1</sub> proteins in *Rhodospirillum rubrum*. *J. Bacteriol.* **189**:6861–6869.
72. **Zhang, Y., R. H. Burris, P. W. Ludden, and G. P. Roberts.** 1995. Comparison studies of dinitrogenase reductase ADP-ribosyl transferase/dinitrogenase reductase activating glycohydrolase regulatory systems in *Rhodospirillum rubrum* and *Azospirillum brasilense*. *J. Bacteriol.* **177**:2354–2359.
73. **Zhang, Y., R. H. Burris, P. W. Ludden, and G. P. Roberts.** 1993. Posttranslational regulation of nitrogenase activity by anaerobiosis and ammonium in *Azospirillum brasilense*. *J. Bacteriol.* **175**:6781–6788.
74. **Zhang, Y., R. H. Burris, P. W. Ludden, and G. P. Roberts.** 1997. Regulation of nitrogen fixation in *Azospirillum brasilense*. *FEMS Microbiol. Lett.* **152**:195–204.
75. **Zhang, Y., M. Morar, and S. E. Ealick.** 2008. Structural biology of the purine biosynthetic pathway. *Cell Mol. Life Sci.* **65**:3699–3742.
76. **Zhang, Y., E. L. Pohlmann, M. C. Conrad, and G. P. Roberts.** 2006. The poor growth of *Rhodospirillum rubrum* mutants lacking P<sub>II</sub> proteins is due to an excess of glutamine synthetase activity. *Mol. Microbiol.* **61**:497–510.
77. **Zhang, Y., E. L. Pohlmann, C. M. Halbleib, P. W. Ludden, and G. P. Roberts.** 2001. Effect of P<sub>II</sub> and its homolog GlnK on reversible ADP-ribosylation of dinitrogenase reductase by heterologous expression of the *Rhodospirillum rubrum* dinitrogenase reductase ADP-ribosyl transferase-dinitrogenase reductase-activating glycohydrolase regulatory system in *Klebsiella pneumoniae*. *J. Bacteriol.* **183**:1610–1620.
78. **Zhang, Y., E. L. Pohlmann, P. W. Ludden, and G. P. Roberts.** 2001. Functional characterization of three GlnB homologs in the photosynthetic bacterium *Rhodospirillum rubrum*: roles in sensing ammonium and energy status. *J. Bacteriol.* **183**:6159–6168.
79. **Zhang, Y., E. L. Pohlmann, P. W. Ludden, and G. P. Roberts.** 2000. Mutagenesis and functional characterization of the *glnB*, *glnA*, and *nifA* genes from the photosynthetic bacterium *Rhodospirillum rubrum*. *J. Bacteriol.* **182**:983–992.
80. **Zhang, Y., E. L. Pohlmann, P. W. Ludden, and G. P. Roberts.** 2003. Regulation of nitrogen fixation by multiple P<sub>II</sub> homologs in the photosynthetic bacterium *Rhodospirillum rubrum*. *Symbiosis* **35**:85–100.
81. **Zhang, Y., E. L. Pohlmann, and G. P. Roberts.** 2004. Identification of critical residues in GlnB for its activation of NifA activity in the photosynthetic bacterium *Rhodospirillum rubrum*. *Proc. Natl. Acad. Sci. USA* **101**:2782–2787.
82. **Zhang, Y., D. M. Wolfe, E. L. Pohlmann, M. C. Conrad, and G. P. Roberts.** 2006. Effect of AmtB homologs on the posttranslational regulation of nitrogenase activity in response to ammonium and energy signals in *Rhodospirillum rubrum*. *Microbiology* **152**:2075–2089.
83. **Zhu, Y., M. C. Conrad, Y. Zhang, and G. P. Roberts.** 2006. Identification of *Rhodospirillum rubrum* GlnB variants that are altered in their ability to interact with different targets in response to nitrogen-status signals. *J. Bacteriol.* **188**:1866–1874.