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Uridylylation of the P_{II} Protein in the Photosynthetic Bacterium *Rhodospirillum rubrum*

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The regulatory protein P_{II} has been studied in great detail in enteric bacteria; however, its function in photosynthetic bacteria has not been clearly established. As a number of these bacteria have been shown to regulate nitrogenase activity by a metabolic control system, it is of special interest to establish the role of P_{II} in these diazotrophs. In this study, we show that P_{II} in *Rhodospirillum rubrum* is modified in response to the N status in the cell and that addition of ammonium or glutamine leads to demodification. We also provide evidence that P_{II} is uridylylated. In addition, we show that not only these compounds but also NAD^+ promotes demodification of P_{II}, which is of particular interest as this pyridine nucleotide has been shown to act as a switch-off effector of nitrogenase. Demodification of P_{II} by ammonium or NAD^+ did not occur in cultures treated with an inhibitor of glutamine synthetase (methionine sulfoximine), whereas treatment with the glutamate synthase inhibitor 6-diazo-5-oxo-norleucine led to total demodification of P_{II} without any other addition. The results indicate that P_{II} probably is not directly involved in darkness switch-off of nitrogenase but that a role in ammonium switch-off cannot be excluded.

The P_{II} protein is an important regulatory signal both for the metabolic regulation of glutamine synthetase and for transcriptional regulation of NtrC-dependent promoters. In enteric bacteria, P_{II} has been shown to be a trimer of identical subunits, encoded by *glnB* (27, 31). The uridylyltransferase/uridyl-removing enzyme, encoded by *glnD*, acts as a sensor of the intracellular glutamine pool, catalyzing uridylylation of P_{II} at low glutamine concentrations and deuridylylation at high glutamine concentrations (17). Unmodified P_{II} stimulates the enzyme adenylyltransferase, encoded by *glnE*, to catalyze inactivation of glutamine synthetase by adenylylation. P_{II}-UMP promotes the deadenylylation activity of the enzyme, leading to activation of glutamine synthetase (16, 27).

P_{II} also has an important function in the system, controlling transcription from NtrC-activated promoters (13, 20). These promoters regulate transcription from genes encoding proteins involved in nitrogen metabolism, e.g., *glnA*, the structural gene of glutamine synthetase. Transcription from these promoters is activated by the phosphorylated form of NtrC (22). Phosphorylation/dephosphorylation of NtrC is regulated by the status of the P_{II} protein, as unmodified P_{II} interacts with NtrB, thereby eliciting the phosphatase activity of this bifunctional enzyme. The interaction between P_{II} and NtrB is stimulated by α-ketoglutarate and ATP, which both bind to P_{II} (17). The uridylylated form of P_{II} does not interact with NtrB, which then acts as a kinase, phosphorylating NtrC.

In both *Escherichia coli* and *Azospirillum brasilense*, a second copy of the *glnB* gene has been identified, but the in vivo functions of the gene products have not been determined (5, 30). In *A. brasilense* P_{II}, the *glnB* product also has another important function in controlling the activity of NifA, the activator of transcription of other *nif* genes (2).

P_{II} in the cyanobacterium *Synechococcus* sp. strain PCC 7942 has been shown not to be uridylylated, but instead phosphorylation on Ser 49 has been demonstrated (9). The phosphorylating system responds to the cellular state of nitrogen assimilation relative to CO_2 fixation (8).

In the photosynthetic purple bacterium *Rhodospirillum rubrum*, nitrogenase activity is regulated at the metabolic level by reversible ADP-ribosylation of Arg 101 on one subunit of dinitrogenase reductase (19). This modification is catalyzed by dinitrogenase reductase ADP-ribosyltransferase (DRAT). The inhibition of nitrogenase activity has been termed switch-off (34). Nitrogenase activity is recovered as the ADP-ribose moiety is removed in a reaction catalyzed by dinitrogenase reductase glycohydrolase (DRAG). Effectors which cause switch-off are NH_4^+, glutamine, asparagine, darkness, oxygen, carbonyl cyanide *m*-chlorophenylhydrazone, phenazine methosulfate, and NAD^+ (19, 29).

As in most other diazotrophs, ammonium is assimilated via the glutamine synthetase-glutamate synthase pathway in *R. rubrum* (3, 28). Glutamine synthetase activity also shows a switch-off-like behavior during ammonium switch-off (24). We have previously presented data on the complex regulation of transcription of the *glnBA* operon in *R. rubrum* (15). Furthermore, in an *R. rubrum ntrBC* mutant (33), the expression of *glnB* and *glnA* is decreased and nitrogenase switch-off is altered.

Although the DRAT/DRAG system has been characterized at the molecular level, the signal(s) controlling the activities of these enzymes in response to switch-off effectors has not been identified. As P_{II} has been shown to play a major role in regulation of nitrogen metabolism and especially nitrogen fixation, it could be envisaged that P_{II} is involved, directly or indirectly, in controlling DRAT/DRAG. Although *glnB* has been studied to some extent in *Rhodobacter capsulatus* (11), there are no studies of P_{II} at the protein level in photosynthetic bacteria. In this report, we show that P_{II} is modified, presumably by uridylylation. We have also investigated the status of P_{II} during nitrogen-fixing, nitrogen starvation, and different switch-off conditions with respect to the modification.

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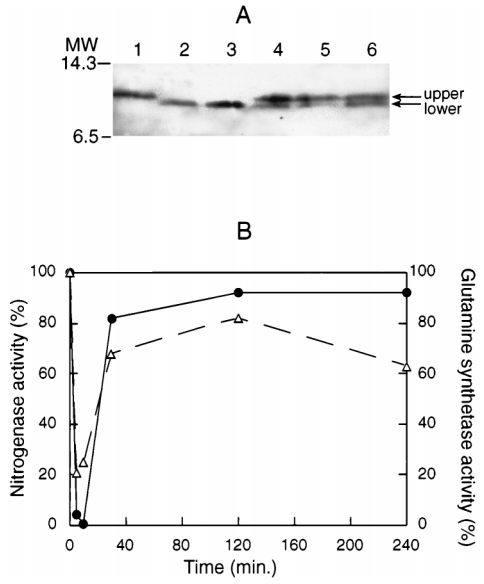

FIG. 1. P_{II} mobility on SDS-PAGE compared with nitrogenase and glutamine synthetase activities during ammonia switch-off. Nitrogen fixing-cultures at an OD_{600} of 1.8 were used. Samples were taken at times specified after addition of 0.5 mM NH_4Cl (final concentration) and treated as described in Materials and Methods. P_{II} was visualized by immunoblotting. (A) P_{II} mobility. Lane 1, no addition; lane 2, 5 min; lane 3, 10 min; lane 4, 30 min; lane 5, 120 min; lane 6, 240 min. Molecular weights (MW) are indicated in thousands. (B) Nitrogenase (●) and glutamine synthetase activities (△) (100% nitrogenase activity = 2,200 nmol of ethylene formed/min/mg of protein; 100% glutamine synthetase activity = 1,300 nmol of γ-glutamylhydroxamate formed/min/mg of protein).

MATERIALS AND METHODS

Bacterial strain and growth conditions. *R. rubrum* S1 was grown in the minimal medium described by Ormerod et al. (26), with the omission of glutamate, but gassed with 95% N_2–5% CO_2. To obtain nitrogen starvation, the gas mixture was changed to 95% argon–5% CO_2 6 h before initiation of the experiments.

P_{II} antiserum preparation and P_{II} detection. We synthesized two peptides, corresponding to amino acids 1 to 15 (MKKIEAIIKPFKLDE-Cys amide) and 48 to 60 (GAEYVVDFLPKVK-Cys amide) of the amino acid sequence deduced from *glnB*. These peptides were used to generate antiserum in rabbits against P_{II}.

To detect P_{II} from *R. rubrum*, cells were precipitated by adding 5.5% trichloroacetic acid and 30% ethanol (final concentrations) and washed three times with 20% 0.3 M Tris-HCl (pH 8)–80% ethanol. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 18% polyacrylamide gels and blotted onto Immobilon-P transfer membranes (Millipore). The two peptide antibodies and an antibody against P_{II} from the cyanobacterium *Synechococcus* sp. strain PCC 7942 (10) were used for identification of P_{II} after visualization by enhanced chemiluminescence (Amersham).

P_{II} modification during diazotrophic growth and switch-off. Experiments were done with cultures grown to an optical density at 600 nm (OD_{600}) of 1 to 1.8. Switch-off effectors were added to 1 mM (final concentration) if not otherwise indicated in the figure legends. Effector solutions added were evacuated and gassed with nitrogen to prevent oxygen effects, e.g., inactivation of nitrogenase. The effects on P_{II} modification of oxygen or darkness were also studied.

To inhibit glutamine synthetase, cultures were incubated for 30 min with 500 μM methionine sulfoximine (MSX). Glutamate synthase was inhibited by incubating the culture for 30 min with 50 μM 6-diazo-5-oxo-norleucine (DON).

Enzymatic and chemical in vitro treatment of modified P_{II}. Precipitated *R. rubrum* proteins containing modified P_{II} were resuspended in 50 mM morpholinepropanesulfonic acid–10 mM $MgCl_2$ (pH 8.6), 1 U of alkaline phosphatase or 0.1 U of snake venom phosphodiesterase was added, and the cultures were incubated at 37°C for 1 h. RNase A treatment (50 μg/ml) was performed on proteins resuspended in 10 mM Tris-HCl–1 mM EDTA (pH 8). The mixtures were incubated 1 h at room temperature.

For chemical treatment, samples were dissolved in 25 mM sodium acetate (pH 6), whereafter 0.1 M (final concentration) HCl or NH_2OH (pH 7) was added. The mixtures were incubated for 3 h at 37°C.

In vivo labeling with [^{32}P]orthophosphate, [5,6-^{3}H]uracil, or [8-^{3}H]adenine. For ^{32}P labeling, cultures were resuspended in medium with no phosphate, supplemented with 1 μCi of [^{32}P]orthophosphate per ml, and incubated for 12 to 14 h. In the tritium labeling experiments, cultures were supplemented with 10 μCi of [5,6-^{3}H]uracil or [8-^{3}H]adenine per ml and grown in standard medium for 24 h. Samples were precipitated before and after addition of 1 mM NH_4Cl. After treatment with 50 μg of RNase A per ml, duplicates of the different samples were subjected to SDS-PAGE and the proteins were transferred to membranes by Western blotting. P_{II} was visualized by autoradiography and immunodetection. Gels with ^{3}H-labeled samples were treated with the fluorographic reagent Amplify (Amersham) before autoradiography for 2 weeks at −70°C with preflashed film. The position of P_{II} was determined in relation to those of radiolabeled molecular weight standards (Rainbow marker; Amersham).

Enzyme assays. Glutamine synthetase activity was determined by the transferase assay as described previously (12). Protein concentrations were measured with the Bio-Rad protein assay. Nitrogenase activity was measured by the acetylene reduction assay (23).

RESULTS AND DISCUSSION

Two forms of P_{II}. Uridylylation of P_{II} has previously been demonstrated in *E. coli* (16, 27), *Klebsiella pneumoniae* (6), *A. brasilense* (5), and *Rhizobium leguminosarum* (4). In the cyanobacterium *Synechococcus* sp. strain PCC 7942, it was shown that P_{II} was phosphorylated on Ser 49 even though it contains the conserved uridylylation site Tyr 51 (9). P_{II} from *R. rubrum* and that from the other *glnB* genes from photosynthetic bacteria sequenced contain the Tyr 51 residue but have an alanine as residue 49 (15).

The results in Fig. 1A clearly show two interconvertible forms of P_{II} in *R. rubrum*. Both bands are in the region of 12 to 13 kDa, which is in good agreement with the mass of the P_{II} subunit as calculated from the amino acid sequence deduced from the *glnB* gene (12,406 Da). P_{II} from nitrogen-fixing cultures migrated more slowly than P_{II} from ammonium-grown cells (Fig. 1A). When P_{II} from glutamate-grown cultures was analyzed, a mixture of the two bands appeared (data not shown). Our two different P_{II} antisera as well as the antibody against P_{II} from *Synechococcus* sp. strain PCC 7942 identified the same two bands (data not shown). Therefore, the antibody against the N-terminal peptide was used in all subsequent experiments. The slower migration of P_{II} indicates that P_{II} in *R. rubrum* is modified during nitrogen-fixing conditions.

Changes in P_{II} migration, glutamine synthetase activity, and nitrogenase activity during ammonium switch-off. It has previously been demonstrated that nitrogenase and glutamine synthetase activities show similar switch-off behaviors when ammonium is added to nitrogen-fixing cultures of *R. rubrum* (7). As shown in Fig. 1, the appearance of the lower band for P_{II} correlates with the decrease in nitrogenase and glutamine synthetase activities during rapid NH_4^+ switch-off. After 5 and 10 min, both activities were very low, with essentially all P_{II} in the lower form. At 30 and 120 min, most of the P_{II} was in the upper form and the activities were increasing. At 240 min, although P_{II} was in both forms, glutamine synthetase activity was decreasing but nitrogenase activity remained constant.

Identification of the group modifying P_{II}. Extracts from nitrogen-fixing cultures were independently treated with alkaline phosphatase, snake venom phophodiesterase, RNase A, HCl, or NH_2OH to investigate the chemical nature of the bond between the modification and P_{II} (Fig. 2). Treatment with alkaline phosphatase, which removes the phosphate from P_{II} in

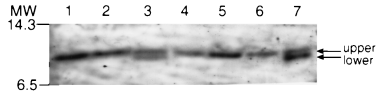

FIG. 2. P_{II} mobility on SDS-PAGE after enzymatic or chemical treatment. The experiment was performed as described in Materials and Methods, and P_{II} was visualized by immunoblotting. Lane 1, control; lane 2, alkaline phosphatase; lane 3, snake venom phosphodiesterase; lane 4, RNase A; lane 5, HCl; lane 6, NH_2OH; lane 7, reference, partially modified P_{II}. Molecular weights (MW) are indicated in thousands.

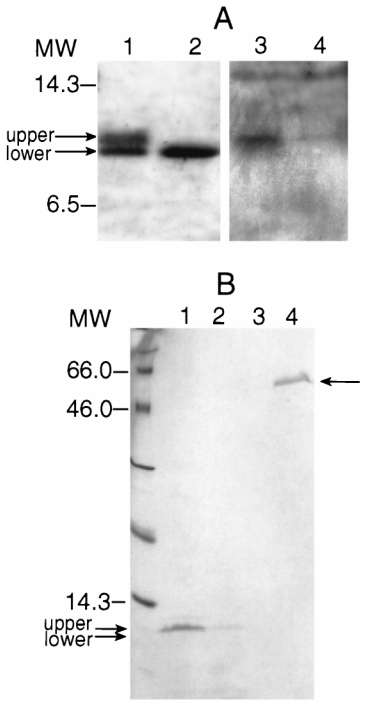

FIG. 3. SDS-PAGE of extracts from cultures grown in the presence of [^{32}P]orthophosphate, [5,6-^{3}H]uracil, or [8-^{3}H]adenine. The experiment was performed as described in Materials and Methods. (A) Extracts from cultures (OD_{600} of 1.5) grown with [^{32}P]orthophosphate. Lanes 1 and 2, immunodetected P_{II} before and after addition of 1 mM (final concentration) NH_4Cl; lanes 3 and 4, autoradiographs of lanes 1 and 2. (B) Extracts from cultures (OD_{600} of 1.3) grown in the presence of [5,6-^{3}H]uracil (lanes 1 and 2) and [8-^{3}H]adenine (lanes 3 and 4). Shown is an autoradiograph of the gel before (lanes 1 and 3) and after (lanes 2 and 4) addition of 1 mM NH_4Cl. The arrow indicates the position of glutamine synthetase. Molecular weights (MW) are indicated in thousands.

Synechococcus sp. strain PCC 7942 (10), had no effect on the migration of the upper P_{II} form. RNase A, which removes 3′-bound nucleotides, also had no effect on P_{II} migration. At least two key enzymes of nitrogen metabolism in *R. rubrum* are modified by ADP-ribosylation: nitrogenase and glutamine synthetase, which can be modified by both adenylylation and ADP-ribosylation (32). Treatment with HCl or NH_2OH is reported to hydrolyze two types of ADP-ribosylations (on glutamate/aspartate and arginine residues) (18), but no change in the migration of P_{II} was detected. However, after treatment with snake venom phosphodiesterase, the lower form of P_{II} appeared. This result clearly indicates that the modifying moiety is bound to the P_{II} protein as a phosphodiester. To further identify the type of P_{II} modification present in *R. rubrum*, nitrogen-fixing cultures were labeled with [^{32}P]orthophosphate, [5,6-^{3}H]uracil, or [8-^{3}H]adenine. After SDS-PAGE of samples from cultures grown with [^{32}P]orthophosphate or [5,6-^{3}H]uracil, labeled proteins identical with the upper of the two P_{II} bands were identified (Fig. 3). The Western blot analysis in Fig. 3A shows that P_{II} is present in two forms under nitrogen-fixing conditions, which is different from the results presented in Fig. 1. This might be due to effects of the phosphate starvation prior to the experiment, which, for example, could lead to a depletion of the UTP pool. Upon addition of NH_4Cl, the ^{32}P- and uracil-labeled P_{II} bands essentially disappeared, in agreement with the results in Fig. 1. In samples from cultures grown under nitrogen-fixing conditions in the presence of [8-^{3}H]adenine, no labeled proteins were detected. However, after addition of NH_4Cl, a single band located at a position equal to that of glutamine synthetase (24, 28) appeared (Fig. 3B). The fact that no adenine-labeled P_{II} could be demonstrated shows that there is no adenylylation or ADP-ribosylation of P_{II} in *R. rubrum.*

The presence of uracil and phosphate in the slower-moving form of P_{II} and the fact that it could be converted to the faster-moving form by treatment with snake venom phosphodiesterase shows for the first time in a photosynthetic bacterium that P_{II} is modified by uridylylation, probably at Tyr 51, as in other organisms.

Changes in P_{II} modification by switch-off effectors. In enteric bacteria, P_{II} is involved in the regulation of glutamine synthetase activity as well as controlling transcription of, e.g., *glnA*. In photosynthetic bacteria, the metabolic regulation of nitrogenase switch-off has been shown to be a complex process with different signals regulating the DRAT/DRAG system (19, 25, 29). To compare nitrogenase switch-off with P_{II} modification, the effects of different effectors were studied. Addition of glutamine caused demodification of P_{II}, and NH_4Cl had a similar effect (Fig. 4). During nitrogen-fixing conditions, the NH_4^+/carbon ratio is low and with glutamine synthetase in its active form; thus, it can be postulated that addition of NH_4^+ leads to a rapid increase in glutamine concentration. This signal causes the uridylyltransferase to catalyze deuridylylation of P_{II}, assuming that uridylyltransferase in *R. rubrum* is similar to the *E. coli* enzyme (17). After addition of glutamate (which is not a switch-off effector), P_{II} was partially demodified (Fig. 4). Even though the total nitrogen level is increased when glutamate is added, the ammonium concentration is possibly not sufficient to increase the glutamine concentration enough to lead to total demodification of P_{II}. Glutamine, NH_4Cl, and glutamate had effects on P_{II} modification in nitrogen-starved cultures (gassed with argon) similar to those in nitrogen-fixing cultures (data not shown).

We have previously reported on nitrogenase switch-off by NAD^+ in *R. rubrum*. Indeed, 1 mM NAD^+ generated the unmodified P_{II} form (Fig. 4), whereas no P_{II} demodification was observed when 1 mM NADH, NADPH, or $NADP^+$ was added (Fig. 5A). In nitrogen-starved cultures, NAD^+ had a very minor effect on the P_{II} modification (Fig. 5B). Changes in the NAD^+ concentration have previously been suggested to play an important role in the nitrogenase switch-off mechanism, probably by directly affecting the DRAT/DRAG system (25, 29). Darkness also acts a nitrogenase switch-off effector. When cultures were transferred from light to darkness, a rapid increase in the NAD^+/NADH ratio was observed (14, 25). However, no effect was seen on P_{II} modification by rapid darkness switch-off, changing the atmosphere to oxygen, or both (Fig. 6). One possible explanation for the NAD^+-induced demodification of P_{II} could be that the NAD-dependent reaction catalyzed by glutamate dehydrogenase, i.e., synthesis of glutamate from α-ketoglutarate and ammonium, is driven backwards by the addition of NAD^+. The ammonium released

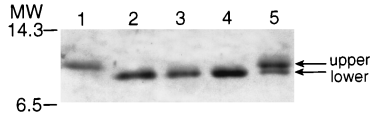

FIG. 4. P_{II} mobility on SDS-PAGE in extracts from cells subjected to switch-off. The experiment was performed as described in Materials and Methods, and P_{II} was visualized by immunoblotting. N_2-fixing cells (OD_{600} of 1.3) were used. Effectors were added at 1 mM (final concentration). Lane 1, control; lane 2, NH_4Cl; lane 3, NAD^+; lane 4, glutamine; lane 5, glutamate. Molecular weights (MW) are indicated in thousands.

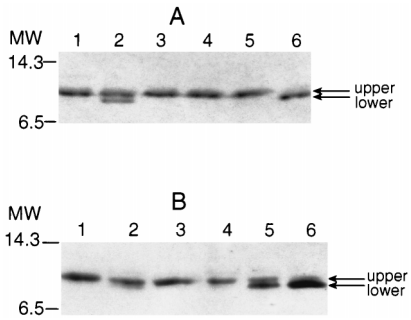

FIG. 5. Effect on P_{II} mobility on SDS-PAGE by addition of NAD(P)(H) in nitrogen-fixing and nitrogen-starved cultures. The experiment was performed as described in Materials and Methods, and P_{II} was visualized by immunoblotting. (A) Nitrogen-fixing cultures. Lane 1, control; lane 2, NAD^+; lane 3, NADH; lane 4, $NADP^+$; lane 5, NADPH; lane 6, NH_4Cl. (B) Lanes 1 to 3, nitrogen-starved cultures; lanes 4 to 6, nitrogen-fixing cultures. Lanes 1 and 4, no addition; lanes 2 and 5, 5 mM NAD^+ incubated for 1 min; lanes 3 and 6, 5 mM NAD^+ incubated for 10 min. All cultures used were grown to an OD_{600} of 1.6. Molecular weights (MW) are indicated in thousands.

could then react with glutamate, forming glutamine, which would support P_{II} demodification. During nitrogen starvation, the substrate level (glutamate) is probably too low for the reaction to occur.

Demodification of P_{II} is affected in cultures treated with MSX and DON. NH_4^+ or NAD^+ addition did not cause demodification of P_{II} in cultures treated with MSX, an inhibitor of glutamine synthetase, during nitrogen-fixing conditions. In contrast, glutamine addition did cause demodification of P_{II} in MSX-treated cells (Fig. 7). The fact that glutamine was the only switch-off effector causing P_{II} deuridylylation indicates that the uridylyltransferase activity in *R. rubrum*, as in enteric bacteria (17), responds to the intracellular glutamine concentration. When the glutamate synthase inhibitor DON was added, P_{II} was converted to its demodified form even in N_2-grown cells, probably due to an increase in the glutamine concentration when further assimilation by glutamate synthase was inhibited (data not shown).

Although demodification of P_{II} is concomitant with ammonium switch-off of both nitrogenase and glutamine synthetase and P_{II} is demodified by NAD^+, there are differences between P_{II} deuridylylation and increased DRAT activity. In the MSX-treated culture, glutamine was clearly affecting P_{II} modification; however, glutamine addition to these cultures did not lead to typical nitrogenase switch-off (data not shown). We have previously reported that $NADP^+$ acts as a switch-off

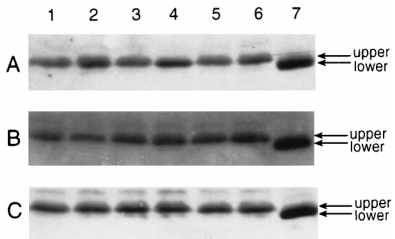

FIG. 6. Effect on P_{II} mobility on SDS-PAGE by darkness and/or oxygen. Samples were taken at the times indicated after subjecting cells to darkness (A), gassing the culture with oxygen (B), or both (C) and were treated as described in Materials and Methods. P_{II} was visualized by immunoblotting. Lane 1, before treatment; lane 2, 1.0 min; lane 3, 2 min; lane 4, 4 min; lane 5, 8 min; lane 6, 16 min; lane 7, reference, unmodified P_{II}. All cultures used were grown to an OD_{600} of 1.2.

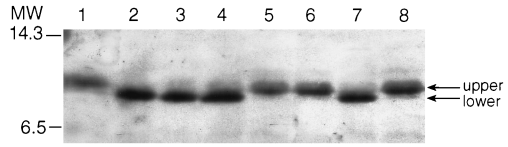

FIG. 7. Effect on P_{II} mobility on SDS-PAGE by switch-off effectors in the presence of MSX. The experiment was performed as described in Materials and Methods, and P_{II} was visualized by immunoblotting. N_2-fixing cultures grown to an OD_{600} of 1.2 were used. Lanes 1 to 4, control with no MSX added; lanes 5 to 8, 500 μM MSX added and incubated for 30 min before addition of effector. Lanes 1 and 5, no effector added; lanes 2 and 6, NH_4Cl; lanes 3 and 7, glutamine; lanes 4 and 8, NAD^+. Molecular weights (MW) are indicated in thousands.

effector in *R. rubrum* (29); however, as shown here, it did not have any effect on P_{II} modification. Yet another difference is the fact that NAD^+ but not NH_4^+ caused switch-off in nitrogen-starved cultures (28), which is in contrast to our results on the modification of P_{II}. It has previously been suggested that there are different systems controlling DRAT activity, one responding to the nitrogen status and one responding to the energy status (33). In an *R. rubrum ntrBC* mutant, where the expression of *glnB* is much lower than in wild-type cells (15), ammonium switch-off was abolished although switch-off caused by darkness was less affected (33). These observations indicate that P_{II} possibly affects DRAT activity when NH_4^+ is the switch-off effector but not during darkness switch-off. In this investigation, we could not detect P_{II} demodification by darkness or oxygen.

In summary, we have presented evidence showing that addition of ammonium or glutamine leads to a change of the uridylylation state of P_{II} in *R. rubrum* and that a role of P_{II} as a signal transducer in ammonium switch-off cannot be excluded. To further investigate the physiological role(s) of P_{II} in *R. rubrum*, we have made a number of attempts to generate *glnB* mutants but have so far been unsuccessful. This could be because such mutations are lethal or, alternatively, because it is difficult to obtain a high recombination frequency in the *glnB* region as has been reported for some *Rhizobium* species (1, 21). A *glnB* mutant would clearly be instrumental in establishing if P_{II} in *R. rubrum* has a role in the switch-off effect and if it is involved in regulation of glutamine synthetase activity and/or in transcriptional regulation of nitrogen fixation. In this context, the observation that P_{II} regulates NifA activity in *A. brasilense* is of special interest, especially as the identity between the P_{II} proteins from *R. rubrum* and *A. brasilense* is very high (15).

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