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The P_{II} Protein in the Cyanobacterium *Synechococcus* sp. Strain PCC 7942 Is Modified by Serine Phosphorylation and Signals the Cellular N-Status

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The *glnB* gene product (P_{II} protein) from *Synechococcus* sp. has previously been identified among ^{32}P-labeled proteins, and its modification state has been observed to depend on both the nitrogen source and the spectral light quality (N. F. Tsinoremas, A. M. Castets, M. A. Harrison, J. F. Allen, and N. Tandeau de Marsac, Proc. Natl. Acad. Sci. USA 88:4565–4569, 1991). As shown in this study, modification of the P_{II} protein primarily responds to the N-status of the cell, and its light-dependent variations are mediated through nitrate metabolism. Modification of the P_{II} protein results in the appearance of three isomeric forms with increasing negative charge. Unlike its homolog counterparts characterized so far, P_{II} in *Synechococcus* sp. is modified by phosphorylation on a serine residue, which represents a unique kind of protein modification in bacterial nitrogen signalling pathways.

Cyanobacteria are characterized by an autotrophic mode of growth, performing oxygenic photosynthesis. Their simple nutritional requirements, together with highly evolved capacities of adaptation to changing environmental conditions, enable them to colonize nearly all ecosystems (for a review see reference 27). Whereas carbon is primarily assimilated from CO_2, nitrogen is acquired, depending on its availability, as ammonium, nitrite, nitrate, and in the case of diazotrophic cyanobacteria, molecular nitrogen. The different nitrogen sources are utilized in a hierarchical order (ammonium > nitrate > N_2), which parallels the increasing energy requirement for their assimilation (reviewed in reference 7). Moreover, nitrogen assimilation is tightly coupled to the fixation of CO_2 (7, 10, 20). By inhibiting the ammonia-assimilating enzyme glutamine synthetase, nitrate reduction can be uncoupled from CO_2 fixation. Although these data suggest that glutamine formation is of central importance in nitrogen regulation, the molecular basis of nitrogen control has not yet been identified.

Interactions between the regulatory processes controlling nitrogen metabolism and photosynthesis have had limited investigation. Nitrogen-deprived cells exhibit reduced photosynthetic capacities; this is primarily caused by the degradation of phycobilisomes, which serve as light-harvesting antennae in cyanobacteria (27). The green alga *Selenastrum minutum* responds to the different demand for ATP and reducing equivalents when growing on nitrate or ammonia by modulating the distribution of excitation energy between photosystem I (PS I) and photosystem II (PS II) (state transitions) (29). Such a mechanism has not yet been shown to occur in cyanobacteria.

Recently, a *glnB* homolog gene from *Synechococcus* sp. strain PCC 7942 has been cloned and sequenced (28). Its gene product, called the P_{II} protein, was previously identified in the closely related strain *Synechococcus* sp. strain PCC 6301 from in vivo ^{32}P-labeling experiments (8, 22). P_{II} was shown to be highly modified in the presence of nitrate and in response to light favoring PS II excitation (PS II light), while light favoring PS I (PS I light) and the presence of ammonium led to a decreased level of modification (28). In enteric bacteria, P_{II} is known as a central signal transmitter of the N-status of the cell (17, 24). The bifunctional GlnD protein uridylylates P_{II} under nitrogen-limiting conditions and deuridylylates P_{II}-UMP in the excess of nitrogen (1). P_{II} modification is monitored by the adenylyltransferase-adenylyl-removing enzyme, which regulates glutamine synthetase activity (11), and by the sensor kinase NtrB, which transmits its signal to the response regulator NtrC (16). The finding that P_{II} modification in *Synechococcus* sp. strain PCC 6301 was related to the light regimen led to the suggestion that P_{II} might act as a central integrator of nitrogen control and light-dependent regulation (28).

We show here that modification of P_{II} in *Synechococcus* sp. responds primarily to the cellular N-status, whereas illumination influences its modification state indirectly. We also present evidence that the cyanobacterial P_{II} protein is modified by O phosphorylation rather than uridylylation as occurs in other eubacteria.

MATERIALS AND METHODS

Materials. Restriction enzymes were obtained from New England Biolabs or Boehringer Mannheim; phosphodiesterase I (*Crotalus adamanteus* venom) was purchased from Pharmacia; calf intestinal alkaline phosphatase was from Promega. Radiochemicals were from Amersham, 30% (wt/vol) acrylamide–0.8% (wt/vol) bisacrylamide stock solution was a product of National Diagnostics, and molecular mass standard proteins for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were purchased from Bio-Rad Laboratories. Size standards for nondenaturating PAGE were from Pharmacia. Other chemicals were obtained from Sigma or Merck.

Culture conditions. *Synechococcus* sp. strain PCC 7942 spc. (9) (hereafter designated *Synechococcus* sp. strain PCC 7942)

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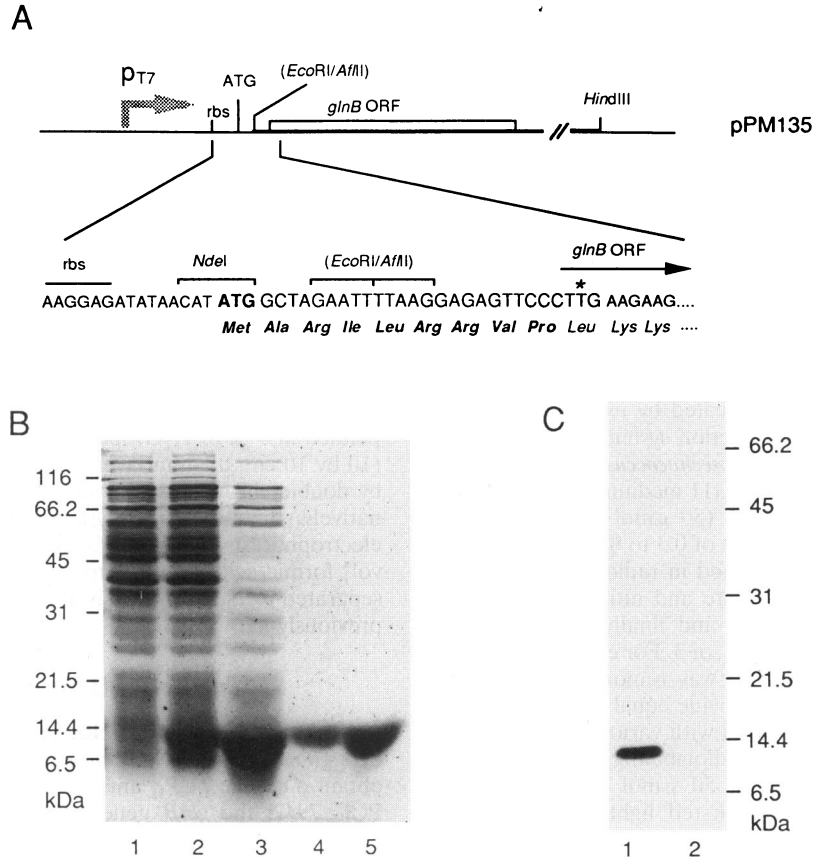

FIG. 1. (A) Schematic representation of the *glnB* gene in plasmid pPM135 together with the upstream phage T7 promoter (p_{T7}) and translational initiation signals derived from vector pT7-7 (26); rbs, ribosomal binding site; ORF, open reading frame. The nucleotide sequence around the translational initiation site and the derived amino acid sequence of the N-terminal part of the corresponding gene product are given below. Amino acids in boldface are N-terminal extensions of P_{II}* derived from the construction and not present in the wild-type P_{II} protein. The asterisk indicates the TTG initiation codon of the wild-type *glnB* gene in *Synechococcus* sp. strain PCC 7942. (B) Overexpression of P_{II} in *E. coli* BL21 and purification of the protein shown by SDS-PAGE and Coomassie blue staining of the proteins. Lanes: 1, SDS lysate of cells of strain BL21(pPM135) before induction; 2, SDS lysate of cells of strain BL21(pPM135) after induction of the T7 RNA polymerase; 3, fraction of P_{II}* purification, polyethylene glycol 6000-precipitated ammonium sulfate fraction; 4 and 5, purified P_{II}* protein, 2 and 6 μg, respectively. (C) Reactivity of the P_{II}-specific antibodies in immunoblot analysis. Cell lysates were separated by SDS-PAGE, the proteins were transferred to nitrocellulose and incubated with the antiserum. Cross-reactive antibody localization was achieved with the enhanced chemoluminescence detection system. Lanes 1, *Synechococcus* sp. strain PCC 7942; 2, $GlnB^-$ mutant from *Synechococcus* sp. strain PCC 7942.

and strain PCC 6301 were grown photoautotrophically at 25°C in liquid BG11 medium (19) supplemented with 10 mM $NaHCO_3$. Either 18 mM $NaNO_3$ or 5 mM NH_4Cl buffered with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5) was used as the nitrogen source. Cells of *Escherichia coli* were grown in LB medium (21); transformants carrying pT7-7-derived vectors (26) were selected on ampicillin (80 μg/ml).

Cloning techniques and genetic methods. Standard DNA manipulation methods were performed as described by Sambrook et al. (21).

Construction of the *glnB* high-level expression vector pPM135 and purification of the hybrid P_{II} protein. Plasmid pPM119 carrying the *glnB* gene from *Synechococcus* sp. strain PCC 7942 (28) was digested with *Afl*II, and the ends were filled in by using the Klenow fragment of DNA polymerase I. The linearized plasmid was cut with *Hin*dIII, and the 3.7-kb fragment containing the *glnB* gene was recovered. Vector pT7-7 (26) was restricted with *Eco*RI, the ends were filled in as described above, and the DNA was then digested with *Hin*dIII; this preparation of vector DNA was ligated to the 3.7-kb *Afl*II–Klenow-filled-in *Hin*dIII fragment containing *glnB* to give plasmid pPM135 (Fig. 1). This plasmid directs the synthesis of a hybrid P_{II} protein, P_{II}*, which contains nine amino acids fused to the N terminus of the intact P_{II} sequence. Plasmid pPM135 was used to transform cells of *E. coli* BL21 (25). Production of the P_{II}* protein was induced by adding isopropylthiogalactopyranoside (IPTG) to 0.4 mM (final concentration). After 2 h of induction, the cells were harvested by centrifugation. Wet cell paste of 5 g was resuspended in 15 ml of buffer I (50 mM Tris Cl [pH 7.5], 75 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 μg of phenylmethylsulfonyl fluoride per ml). Cells were broken by two consecutive passages through a French pressure cell at 16,000 lb/in^2. Cell debris was removed by centrifugation at 20,000 × *g* for 15 min. The supernatant was clarified by addition of protamine sulfate to 0.2% (wt/vol) followed by centrifugation at 20,000 × *g*. Ammonium sulfate was added to the supernatant to give 33% saturation; after centrifugation, the soluble fraction was adjusted to 50% ammonium sulfate saturation. The precipitate was recovered, dissolved in buffer I, and desalted on a Sephadex G-50 column equilibrated with the same buffer. Polyeth-

ylene glycol 6000 was then added to 12.5% (wt/vol) (final concentration); the precipitate was collected and resuspended in buffer II (50 mM Tris Cl [pH 8.0], 60 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA) and passed through a DEAE-Sephacel column (1.5 by 6 cm) equilibrated with the same buffer. The flowthrough contained about 10 mg of electrophoretically homogeneous P_{II}* protein.

Immunological procedures. For the generation of a P_{II}-specific antiserum, a rabbit was immunized by intradermal injections of purified P_{II}* protein by the method of Danchin et al. (4) except that incomplete Freund's adjuvant replaced the complete adjuvant. Crude immunoglobulin G fraction was prepared by 33% ammonium sulfate precipitation (2). For immunoblot experiments (21), proteins were blotted on nylon-supported nitrocellulose (Hybond C super; Amersham), and cross-reactive antibodies were located by using an enhanced chemoluminescent antibody detection system (Amersham).

In vivo phospholabeling of *Synechococcus* sp. strain PCC 7942 cells. Cells were grown in BG11 medium and illuminated with cool white fluorescent light (50 μmol m^{-2} s^{-1}) to an optical density at 750 nm (OD_{750}) of 0.3 to 0.4. The cells were harvested by centrifugation, washed in radiolabeling medium (BG11 medium without phosphate and nitrate and buffered with 10 mM HEPES [pH 7.5]), and finally resuspended in radiolabeling medium to an OD_{750} of 3. For each radiolabeling sample, 1 ml of cell suspension was removed in a 35-mm-diameter tissue culture dish to provide equal illumination, and the suspension was supplemented with various compounds as indicated. Illumination during radiolabeling was provided by cool white fluorescent lights at 50 μmol m^{-2} s^{-1} unless otherwise stated. Orange and far-red light for preferential excitation of PS II and PS I, respectively, were obtained as described previously (28). Carrier-free $^{32}P_i$ (30 μCi) was added to each sample; after 90 min of incubation, the cell suspension was acidified with trichloroacetic acid (TCA) (5% [wt/vol], final concentration). The cells were pelleted, washed with 90% (vol/vol) acetone, and lysed in 160 μl of SDS sample buffer (21) by heating to 90°C for 5 min. A 40-μl aliquot of each sample was subjected to SDS-PAGE followed by blotting on nitrocellulose (Hybond C super) with use of a semidry electroblotting device (Prolabo). The membrane was washed for 10 min in distilled water and stained with 0.1% (wt/vol) Ponceau S in 3% (wt/vol) TCA. Residual background radioactivity was removed by incubating the blot for 60 min in 20% TCA at 65°C. The blot was autoradiographed, and the P_{II} protein was detected immunologically.

Determination of P_{II} modification by PAGE under nondenaturing conditions followed by immunoblotting. Cells were adjusted to an OD_{750} of 2.5 and acclimated under the appropriate conditions for 1 h unless otherwise stated. A 2-ml sample of cell suspension was removed in a 2-ml test tube and chilled for 5 s in liquid N_2, and the cells were harvested by centrifugation at 4°C. The cells were resuspended in 200 μl of 50 mM Tris Cl (pH 7.5)–4 mM EDTA–0.5 mM phenylmethylsulfonyl fluoride; about 100 μl of glass beads (0.11-mm diameter; Braun) was added, and the cells were broken by three consecutive cycles of vortexing for 1 min followed by freezing in liquid N_2. Finally, cell debris was removed by centrifugation, and the supernatant was recovered. A 10-μl sample of cell extract was mixed with 10 μl of loading buffer (50 mM Tris Cl [pH 6.8], 20% [vol/vol] glycerol, 0.005% [wt/vol] bromophenol blue) and subjected to PAGE under nondenaturing conditions, using the high-pH discontinuous buffer system of Davis and Ornstein as described by Goldenberg (6), with Nonidet P-40 added to the stacking and separating gels to 0.025% (wt/vol) (final concentration). Following electrophoresis, P_{II} isoforms were revealed by immunoblotting as described above.

Determination of the phosphoamino acid composition of the P_{II} protein. P_{II} protein was radiolabeled in vivo with $^{32}P_i$, and cells were broken by vortexing with glass beads as described above. The P_{II} protein was removed from the cell extract by immunoprecipitation with the P_{II}-specific antibodies and with protein A-Sepharose CL 4B as described previously (21). The washed immunoprecipitate was dissolved in SDS sample buffer, and the proteins were separated by SDS-PAGE. Following electrophoresis, the proteins were transferred to Immobilon (Millipore); the P_{II} protein was detected by autoradiography of the membrane, and the part of the membrane containing the P_{II} protein was cut out. The P_{II} protein bound to Immobilon was hydrolyzed in 6 N HCl at 110°C for 2 h. The hydrolysate was dried in a Speed-Vac concentrator, resuspended in 4 μl of H_2O, spotted on a cellulose thin-layer plate (10 by 10 cm; 0.1-mm layer thickness; Merck), and separated by double chromatography as described elsewhere (5). Alternatively, the dried hydrolysate was resuspended in 4 μl of electrophoresis buffer (7.8% [vol/vol] acetic acid, 2.5% [vol/vol] formic acid), applied to a cellulose thin-layer plate, and separated by electrophoresis at 400 V for 40 min as described previously (5).

RESULTS

Generation of a P_{II}-specific antiserum and immunological detection of P_{II} in *Synechococcus* sp. strain PCC 7942. To obtain a source of P_{II} antigen from *Synechococcus* sp. strain PCC 7942, the *glnB* gene originating from this strain was subcloned into *E. coli* expression vectors of the pT7 series. Preliminary experiments showed that translational initiation of that gene was poor in *E. coli*; therefore, the expression vector pT7-7, which contains a strong ribosomal binding site followed by an ATG start codon and a multiple cloning site, was used. The *glnB* coding region was cloned into the *Eco*RI site of pT7-7 in frame with the ATG start codon (Fig. 1A) to give plasmid pPM135. The predicted hybrid gene product should contain an extension of nine amino acids at the N terminus of the P_{II} protein. Induction of pPM135 in *E. coli* BL21 resulted in strong overproduction of a 14-kDa protein (Fig. 1B). We devised a simple three-step purification procedure which resulted in an electrophoretically homogeneous protein preparation (Fig. 1B). To confirm the identity of the purified protein as the putative hybrid *glnB* gene product, we determined its amino acid composition. The result corresponded precisely to that predicted for the hybrid P_{II} protein (termed P_{II}*) as derived from the nucleotide sequence of the gene fusion.

The purified protein was used to raise a P_{II}-specific polyclonal antiserum in rabbits. In immunoblot experiments, antibodies obtained from this serum specifically recognized a protein of 13 kDa in a *Synechococcus* sp. strain PCC 7942 cell lysate (Fig. 1C); this reactivity was absent in the preimmune serum (data not shown). The size of the immune reactive protein corresponds well with the known molecular mass of P_{II} from this strain. Moreover, the band is absent in a mutant of *Synechococcus* sp. strain PCC 7942 in which the *glnB* gene was inactivated by insertion of a kanamycin resistance gene (5a).

Quantitative estimation of the amount of P_{II} in ammonium- and nitrate-grown *Synechococcus* sp. strain PCC 7942 was achieved by using increasing amounts of the purified protein as a standard reference in immunoblot experiments (Fig. 2). In 15 μg of protein in cell lysates, 10 to 20 ng of P_{II} protein, i.e., about 0.1% of the total protein, could be detected. The cellular concentration was approximately the same in nitrate- and

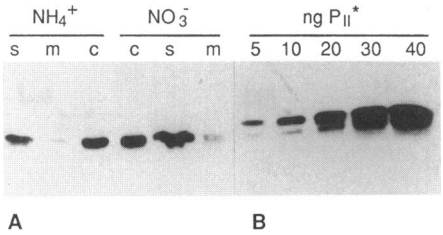

FIG. 2. Quantification and subcellular localization of P_{II} as determined by immunoblotting. (A) Cells of *Synechococcus* sp. strain PCC 7942 were grown in the presence of ammonium or nitrate to mid-exponential phase (OD_{750} = 0.5). Cells were broken by two consecutive passages through a French pressure cell at 16,000 lb/in^2, and cell debris was removed by centrifugation at 10,000 × *g* for 10 min. The resulting crude cell extract (c) was then separated in soluble (s) and membrane (m) fractions by streptomycin sulfate precipitation as described previously (23). The crude cell extracts contained 15 μg of protein; for the soluble and membrane fractions, the corresponding volume equivalents were used. (B) Increasing quantities of P_{II}* protein (5 to 40 ng) were run as a standard reference. A breakdown product corresponding in size to native P_{II} appeared in the P_{II}* preparation after 10 months of storage.

ammonium-grown cells. P_{II} was recovered in the soluble fraction, with only minor amounts detectable in the membrane preparation.

In vivo ^{32}P labeling of the P_{II} protein in *Synechococcus* sp. strain PCC 7942. Initially, we analyzed the modification of P_{II} in *Synechococcus* sp. strain PCC 7942 by in vivo ^{32}P labeling as described previously (28). The cells were shifted to different conditions of spectral light quality and nitrogen sources while incubated in the presence of $^{32}P_i$ for 90 min. To identify P_{II} among the radiolabeled proteins, the cell lysate was separated by electrophoresis and transferred to nitrocellulose, where contaminating nucleic acids could be removed from bound proteins by washing. The membrane could then be used both for autoradiography and for immunological detection of the P_{II} protein. Three major radiolabeled proteins of molecular masses of 60, 30, and 13 kDa could be detected in the cell lysate (Fig. 3). With longer exposure times, additional bands at 55, 22, and 15 kDa appeared (data not shown). The radiolabeled 13-kDa band comigrated with the P_{II} protein, as detected with the P_{II}-specific antibodies. The radiolabeling intensity of P_{II} was quite similar under white, orange (PS II-specific), or far-red (PS I-specific) illumination. The labeling of P_{II} was greatly reduced in the presence of ammonium, irrespective of the incident light. When the cells were incubated with ammonium together with L-methionine sulfoximine, an inhibitor of glutamine synthetase, P_{II} was again modified; its labeling was even greater than that in the nitrate-grown cells. This result showed that the P_{II} modification was primarily governed by the cellular N-status under our assay conditions.

Electrophoretic separation of unmodified and three modified forms of P_{II} protein. There are limitations in determining the modification state of P_{II} by in vivo ^{32}P labeling: the labeling intensity depends not only on the actual state of P_{II} modification but also on the uptake and metabolism of added $^{32}P_i$ and on the turnover rate of modified P_{II} protein. We therefore decided to analyze the modification state of P_{II} by an alternative method. In *E. coli*, P_{II} and P_{II}-UMP could be separated by electrophoresis under nondenaturing conditions (1). Extracts from ^{32}P-labeled cells of *Synechococcus* sp. strain PCC 7942 were subjected to gel electrophoresis under nondenaturing conditions; P_{II} was then detected by immunoblotting, and total

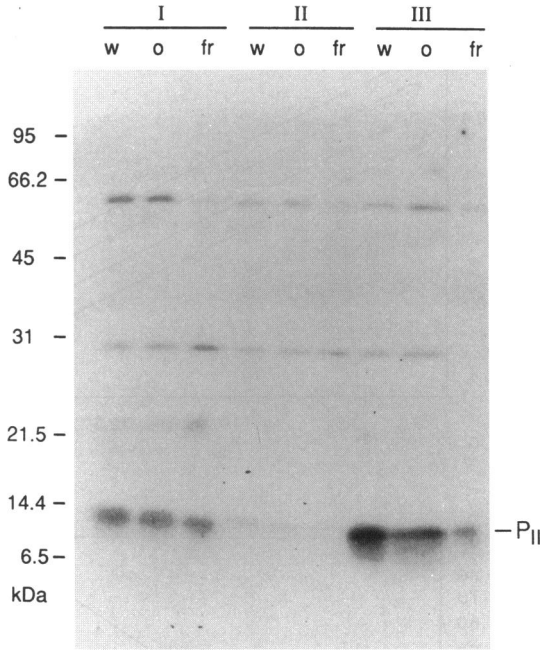

FIG. 3. Autoradiogram of in vivo ^{32}P-labeled proteins of *Synechococcus* sp. strain PCC 7942 separated by SDS-PAGE. Cells were labeled in the presence of 10 mM sodium nitrate (I), 10 mM sodium nitrate and 5 mM ammonium chloride (II), or 10 mM sodium nitrate, 5 mM ammonium chloride, and 0.1 mM L-methionine sulfoximine (III); illumination was with white light (w), orange light (o), or far-red light (fr).

radiolabeled proteins were visualized by autoradiography of the blot (Fig. 4). From ammonium-treated cells, only one band which corresponded to P_{II} was detected immunologically; in addition, three faster-migrating forms of P_{II} could be resolved

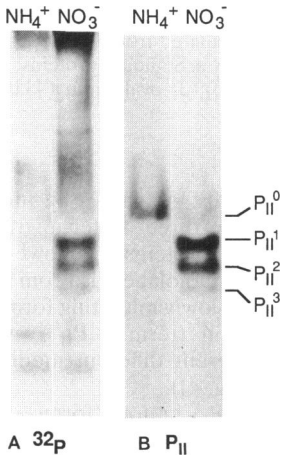

FIG. 4. Separation of unmodified and modified forms of P_{II} by gel electrophoresis under nondenaturing conditions. Extracts of *Synechococcus* sp. strain PCC 7942, which were labeled with $^{32}P_i$ in the presence of 5 mM ammonium chloride or 10 mM sodium nitrate, were prepared as described in Materials and Methods. The proteins were separated by electrophoresis and transferred to nitrocellulose. (A) Autoradiogram of ^{32}P-labeled proteins transferred to nitrocellulose; (B) immunological analysis of the same blot, using the P_{II}-specific antiserum.

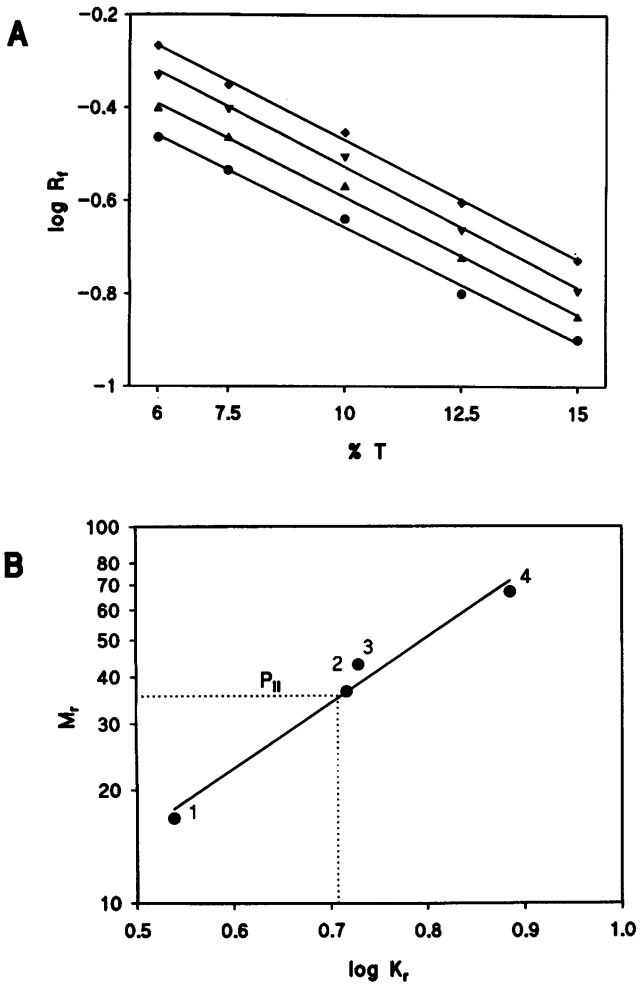

FIG. 5. (A) Ferguson plot of the log mobility (R_f) of the four forms of P_{II} in gels of different-percentage acrylamide (% T). ●, P_{II}^0; ▲, P_{II}^1; ▼, P_{II}^2; ◆, P_{II}^3. (B) Determination of the M_r of native P_{II} protein as described by Neville (15). The retardation coefficient (K_r) of each standard protein was calculated from the slopes of Ferguson plots (s) according to $K_r = -100 \times s$. Standard proteins: 1, myoglobin (16,700); 2, C-phycocyanin (36,500); 3, ovalbumin (43,000); 4, bovine serum albumin (67,000).

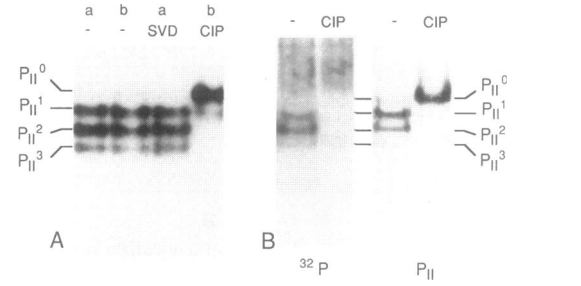

FIG. 6. Enzymatic demodification of modified P_{II} protein. (A) Sensitivity of modified P_{II} protein to phosphodiesterase and alkaline phosphatase. Cell extracts (ca. 10 μg of protein) containing modified forms of P_{II} were incubated in 20 μl of 100 mM Tris Cl (pH 9.0)–10 mM $MgCl_2$ (a) or in 20 μl of 100 mM Tris Cl (pH 9.0)–10 mM $MgCl_2$–1 mM $ZnCl_2$–10 mM spermidine (b) in the absence (−) or presence of 1 U of snake venom phosphodiesterase (SVD) or calf intestinal phosphatase (CIP). After 90 min at 37°C, the modification state of P_{II} was analyzed by nondenaturing gel electrophoresis followed by immunoblotting. (B) Phosphatase treatment of ^{32}P-labeled cell extract containing modified P_{II}. The enzymatic reaction was carried out as described above. The modification state of P_{II} was analyzed by gel electrophoresis under nondenaturing conditions followed by blotting on nitrocellulose. The autoradiogram is shown on the left (^{32}P), and the corresponding immunoblot is shown on the right (P_{II}).

from nitrate-grown cells. As shown by the autoradiogram of the same blot, these three faster-migrating bands corresponded to ^{32}P-labeled proteins; the slowest-migrating form of P_{II}, however, was not radiolabeled. From these results, it can be concluded that the slowly migrating form corresponds to the unmodified P_{II} protein (termed P_{II}^0), whereas the faster-migrating forms represent different modified forms (termed P_{II}^1, P_{II}^2, and P_{II}^3 [Fig. 4]).

The resolution of P_{II} in four forms of different electrophoretic mobility could result from differences in charge or in size. To distinguish between these possibilities, the electrophoretic mobility of P_{II} in gels of different acrylamide concentrations was analyzed by Ferguson plot (15) (Fig. 5A). The slope of the plot is a function of the molecular mass of the protein; the y intercept depends on its charge. As revealed by this analysis, all four forms of P_{II} exhibit the same slope, indicating identical molecular mass. The constant increase in the y intercept shows that the modified forms differ by increasing negative charge.

From a log slope-versus-log M_r plot, P_{II} was determined to migrate as a protein with an M_r of 36,000 in the native gels (Fig. 5B). With the M_r of the P_{II} polypeptide being 12,359 (28), this molecular weight corresponds to a trimeric structure of P_{II}. The three modified forms of P_{II} could thus represent the protein containing one, two, or three modified subunits.

The P_{II} protein from *Synechococcus* sp. strain PCC 7942 is modified by serine phosphorylation. The chemical nature of the modification of the P_{II} protein from *Synechococcus* sp. had not previously been determined. The conservation of the tyrosine residue, which is uridylylated in *E. coli*, and previous reports of phosphodiesterase removal of the radiolabel in P_{II} (8, 28) implied that P_{II} might be uridylylated in *Synechococcus* sp. However, no change in electrophoretic mobility could be observed upon phosphodiesterase treatment of an extract of *Synechococcus* sp. strain PCC 7942 which contained the modified forms of P_{II} (Fig. 6A). Surprisingly, incubation with alkaline phosphatase led to the conversion of the modified forms of P_{II} into P_{II}^0. This result was confirmed by alkaline phosphatase removal of ^{32}P-labeled bands corresponding to P_{II}^1, P_{II}^2, and P_{II}^3, while P_{II}^0 accumulated, as revealed by the immunological analysis of the blot (Fig. 6B).

The result of the demodification of P_{II} by enzymatic treatment indicated that P_{II} is phosphorylated rather than uridylylated in *Synechococcus* sp. strain PCC 7942. Preliminary experiments showed that the ^{32}P-containing modification of P_{II} was resistant to 20% TCA at 80°C, indicative of P_{II} modification through the formation of a phosphomonoester (5). To determine the phosphorylated amino acid, P_{II} protein which had been radiolabeled in cells grown in the presence of nitrate was purified by immunoprecipitation and preparative SDS-PAGE. The P_{II} protein was hydrolyzed with 6 N HCl, and phosphoamino acid analysis was performed by two-dimensional thin-layer chromatography and by thin-layer electrophoresis on cellulose plates (5). The radiolabeled phosphoamino acid was identified as phosphoserine (Fig. 7).

Nitrogen- and light-dependent modification of P_{II} in *Synechococcus* sp. The modification of P_{II} in *Synechococcus* sp.

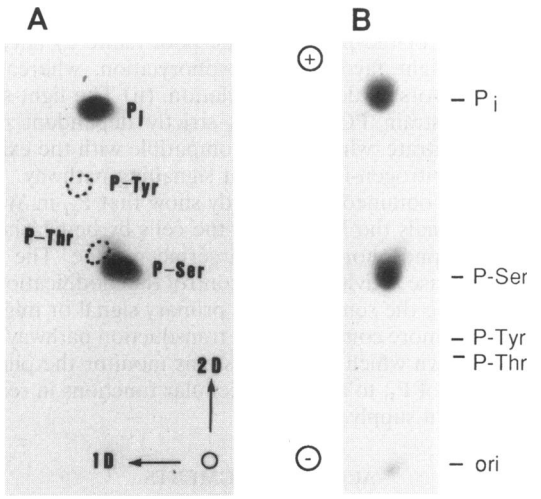

FIG. 7. Analysis of P_{II} phosphoamino acid composition. The hydrolysate of purified ^{32}P-labeled P_{II} protein (ca. 2 μg of protein; 4,000 cpm) was analyzed by two-dimensional thin-layer chromatography (A) or thin-layer electrophoresis (B) followed by autoradiography. Standard phosphoamino acids were coseparated, and their locations were detected by staining with ninhydrin. P-Tyr, phospotyrosine; P-Thr, phosphothreonine; P-Ser, phosphoserine; D, dimension; ori, origin.

under different environmental conditions which are known to cause state 1-state 2 transitions (state of distribution of excitation energy between PS I and PS II) (14) was reinvestigated by using native gel electrophoresis followed by immunoblotting. Cells were grown in the presence of nitrate, and one half of the culture was transferred to nitrogen-free medium. After 1 h of acclimation, both cultures were shifted to darkness under anaerobic conditions for 15 min, to give a complete transition to state 2. The cultures were then divided into aliquots which were placed under white, orange, or far-red light, white light plus 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), or darkness (Fig. 8). Far-red light and white light plus DCMU are correlated with state 1, while orange light and darkness correspond to state 2 conditions (14). In the presence of nitrate, darkness induced partial demodification of the P_{II} protein; upon illumination, P_{II} was modified as before, and the extent of modification was not influenced by the specific light conditions. Under N-limiting conditions, dark incubation was much less efficient in demodifying P_{II}. The modification of P_{II}

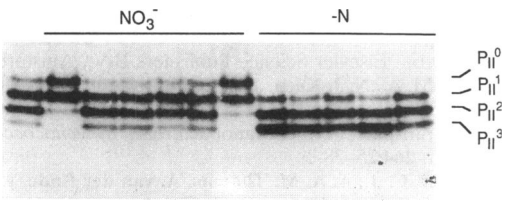

FIG. 8. Modification state of P_{II} in *Synechococcus* sp. strain PCC 7942 as revealed by native PAGE and immunoblotting. Extracts were prepared from cells incubated under the following conditions: nitrate grown, prior to the shift (0); acclimated to darkness and anaerobiosis (I), and then shifted to white light (a), orange light (b), far-red light (c), or white light in the presence of 10 μM DCMU (d), or continued incubation in darkness and anaerobiosis (e), each for 30 min. The shifts were carried out in nitrate-supplemented medium (NO_3^-) and in medium lacking combined nitrogen (−N).

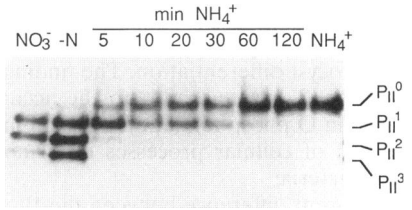

FIG. 9. Response of P_{II} modification to addition of ammonium. Cells grown in the presence of nitrate (NO_3^-) were transferred to nitrogen-free medium (−N) for 1 h. Ammonium chloride was then added to 5 mM (final concentration), and aliquots were removed after 5, 10, 20, 30, 60, and 120 min. For comparison, the modification state of P_{II} in cells grown for several generations in the presence of ammonium is shown (NH_4^+).

was more pronounced in the N-deficient cells than in the presence of nitrate, with P_{II}^2 and P_{II}^3 as the most abundant forms, while the unmodified P_{II} form was absent, independent of the specific light quality. Control experiments using *Synechococcus* sp. PCC 6301 showed that in this strain, the modification of P_{II} was slightly decreased in far-red light and even more decreased in the presence of DCMU when nitrate was present. However, these differences were not observed under conditions of nitrogen starvation (data not shown).

To further investigate the modification of P_{II} in response to the N-status of the cells, its demodification upon the addition of ammonium was examined during a time course experiment. A culture of *Synechococcus* sp. strain PCC 7942, starved for nitrogen for 1 h to yield a highly modified P_{II} protein, was supplemented with ammonium; aliquots were then removed at different intervals and analyzed (Fig. 9). Within 5 min after the addition of ammonium, P_{II}^3 and most of P_{II}^2 disappeared. In contrast, P_{II}^1 was demodified much more slowly. After 120 min, P_{II} was completely demodified, as in cells grown in the presence of ammonium for several generations.

DISCUSSION

In this work, we identified the nature of the P_{II} modification in *Synechococcus* sp. and analyzed factors that control its state of modification. Surprisingly, the P_{II} protein was found to be modified by the formation of a phosphomonoester bond at a serine residue instead of being uridylylated as is characteristic of P_{II} proteins from other gram-negative bacteria (1). The previous report of phosphodiesterase sensitivity of the ^{32}P label in P_{II} from *Synechococcus* sp. PCC strain 6301 (28) might have resulted from alkaline hydrolysis of the phosphomonoester bond. O phosphorylation of regulatory proteins has long been considered to be specific for eukaryotic systems. Signal transduction in bacteria is generally mediated through two-component systems, involving histidine and aspartate protein phosphorylation. To our knowledge, only the phosphotransferase HPr protein from gram-positive bacteria has been identified as a bacterial regulatory protein phosphorylated at a serine residue (18). However, recent findings in different laboratories indicate that protein O phosphorylation may be more common in bacteria than previously thought. A family of putative eukaryotic-like protein kinases has been identified in *Myxococcus xanthus*; at least one of these protein kinases is involved in fruiting body differentiation upon starvation (31). In the cyanobacterium *Anabaena* (*Nostoc*) sp. strain PCC 7120, protein serine kinase activities that are modulated by cellular metabolites have been demonstrated (12). Furthermore, a putative eukaryotic-like protein kinase has been identified

through sequence analysis in *Anabaena* (*Nostoc*) sp. strain PCC 7120 (30); the presumptive gene product could be involved in heterocyst differentiation. The finding of the phosphorylation of the P_{II} protein adds to the accumulating evidence that protein O phosphorylation plays an important role in the regulation of cellular processes in cyanobacteria and possibly other bacteria.

A second apparent difference between the P_{II} protein from proteobacteria and *Synechococcus* sp. concerns the molecular mass of the native proteins. In *E. coli*, P_{II} was shown to be a tetramer; during gel electrophoresis under nondenaturing conditions, only the unmodified and one form of the modified P_{II} protein could be resolved (1). Conversely, P_{II} from *Synechococcus* sp. migrates like a protein of 36 kDa in native gels, which would correspond to a trimeric structure. Consistent with this finding, three isomeric forms of the modified protein could be separated by electrophoresis; they could represent the trimer containing one, two, or three phosphorylated subunits. However, further work is required to definitively establish the quaternary structure of the protein. Whether the different modified isoforms display specific physiological functions also remains to be clarified.

The modification of P_{II} in response to environmental stimuli was investigated by both in vivo ^{32}P-labeling experiments and analysis of P_{II} isoforms. Phosphorylation of P_{II} correlated with the N-status of the cells. In the presence of ammonium, N assimilation through the glutamine synthetase-glutamate synthase cycle is not limited by the nitrogen source; the cells are N replete, and P_{II} is unmodified. Growth on nitrate requires nitrate reduction to ammonium, which is then assimilated. As shown by Coronil et al. (3), under light-limiting conditions, nitrate assimilation and CO_2 fixation compete for reductant and ATP generated by photosynthesis. Therefore, the rate of glutamine formation is substantially lower than in the presence of ammonium (3). Accordingly, P_{II} is predominantly present in its modified forms, P_{II}^1 and P_{II}^2, in nitrate-grown cells. N starvation causes a dramatic decrease in glutamine synthesis (3), with a corresponding stimulation of P_{II} phosphorylation. This correlation with the synthesis of glutamine is further demonstrated by L-methionine sulfoximine inhibition of dephosphorylation of P_{II} upon the addition of ammonium. Therefore, glutamine formation seems to be directly involved in controlling the modification state of P_{II}. In this respect, P_{II} from *Synechococcus* sp. resembles its proteobacterial homologs, in which the state of P_{II} uridylylation is governed by the relative concentrations of glutamine and 2-ketoglutarate.

The modification of P_{II} is independent of a specific light regimen in *Synechococcus* sp. strain PCC 7942. However, incubation in the dark shifted its modification state toward the unmodified form when nitrate was present. Conversely, P_{II} remained modified in the dark when nitrate was absent. This effect can be explained by assuming residual nitrogen assimilation and a concomitant stop of CO_2 fixation in darkness. Consistent with this view, Marqués et al. (13) showed that upon dark incubation of nitrate-grown cells, the pool size of 2-ketoglutarate relative to glutamine decreased threefold in *Synechococcus* sp. strain PCC 6301. Like the demodification of P_{II} in darkness, the decreased modification of P_{II} in cells of *Synechococcus* sp. strain PCC 6301 illuminated with far-red light depends on the presence of nitrate. The different responses in far-red light of *Synechococcus* sp. strains PCC 7942 and PCC 6301 could reflect subtle variations in the efficiency of nitrate assimilation or CO_2 fixation under PS I-specific illumination. Together, these data imply that the signal that governs state 1- state 2 transitions is not involved in the pathway of P_{II} modification. (i) PS II-specific illumination as well as darkness under anaerobiosis are conditions which correspond to a reduced state of the plastoquinone pool (state 2) (14); however, PS II light favors P_{II} phosphorylation, whereas dark incubation favors its dephosphorylation. (ii) The light-specific response in strain PCC 6301 is strictly dependent on the presence of nitrate, which is not compatible with the existence of a second, nitrogen-independent signalling pathway.

The results obtained in this study show that P_{II} in *Synechococcus* sp. signals the N-status of the cells by being phosphorylated or dephosphorylated at a serine residue. The kinase and phosphatase activities which control the modification state of P_{II} might be the sensors of the primary signal or might just be a step in a more complex signal transduction pathway. It has yet to be shown which sensory systems monitor the phosphorylation state of P_{II} to adjust the cellular functions in response to the nitrogen supply.

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