

Protein Tyrosine Phosphorylation in the Cyanobacterium *Anabaena* sp. Strain PCC 7120

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Received 15 November 1996/Accepted 31 January 1997

Components of a protein tyrosine phosphorylation/dephosphorylation network were identified in the cyanobacterium *Anabaena* sp. strain PCC 7120. Three phosphotyrosine (P-Tyr) proteins of 27, 36, and 52 kDa were identified through their conspicuous immunoreactions with RC20H monoclonal antibodies specific for P-Tyr. These immunoreactions were outcompeted completely by free P-Tyr (5 mM) but not by phosphoserine or phosphothreonine. The P-Tyr content of the three major P-Tyr proteins and several minor proteins increased with their time of incubation in the presence of Mg-ATP and the protein phosphatase inhibitors sodium orthovanadate and sodium fluoride. Incubation of the same extracts with [γ - 32 P]ATP but not [α - 32 P]ATP led to the phosphorylation of five polypeptides with molecular masses of 20, 27, 52, 85, and 100 kDa. Human placental protein tyrosine phosphatase 1B, with absolute specificity for P-Tyr, liberated significant quantities of 32 P_i from four of the polypeptides, confirming that a portion of the protein-bound phosphate was present as 32 P-Tyr. Alkaline phosphatase and the dual-specificity protein phosphatase IphP from the cyanobacterium *Nostoc commune* UTEX 584 also dephosphorylated these proteins and did so with greater apparent efficiency. Two of the polypeptides were partially purified, and phosphoamino analysis identified 32 P-Tyr, [32 P]phosphoserine, and [32 P]phosphothreonine. *Anabaena* sp. strain PCC 7120 cell extracts contained a protein tyrosine phosphatase activity that was abolished in the presence of sodium orthovanadate and inhibited significantly by the sulfhydryl-modifying agents *p*-hydroxymercuriphenylsulfonic acid and *p*-hydroxymercuribenzoate as well as by heparin. In *Anabaena* sp. strain PCC 7120 the presence and/or phosphorylation status of P-Tyr proteins was influenced by incident photon flux density.

The phosphorylation and dephosphorylation of proteins on tyrosine constitute a pivotal mechanism for the regulation of enzyme activity and thus cellular functions (27, 29, 30). It was assumed, until only recently, that such protein modifications occurred exclusively within the confines of the eukaryotic cell (15). This assumption was based, in part, on the extensive documentation of phosphohistidine and phosphocarboxyl amino acids in bacterial proteins and the involvement of the latter in a diverse set of bacterial signal transduction mechanisms (24). In fact, tyrosine-phosphorylated proteins may be widespread in representatives of the domain *Bacteria*, although their roles, to date, remain cryptic (4, 5, 14, 19, 26, 31).

The finding of a dual-specificity protein serine/threonine tyrosine phosphatase (IphP) in a cyanobacterium, *Nostoc commune* UTEX 584, raised important questions as to the origin of tyrosine phosphorylation and the evolution of its function (21). Representatives of the cyanobacteria include some of the most morphologically complex of all prokaryotes (6), including filamentous forms which differentiate structurally and biochemically modified heterocysts and resting stages (akinetes 33). For the most part cyanobacteria are obligate photoautotrophs, many have the capacity to fix dinitrogen, and various physiological processes in some strains exhibit circadian rhythms (13). These properties of cyanobacteria, including the phylogenetic significance of these microorganisms, their prevalence in extreme environments, their widespread distribution, and their ecological importance (2), especially their capacity to elaborate peptide toxins which act as potent inhibitors of protein serine/threonine phosphatases (1), make them an attrac-

tive target for the study of the role of tyrosine phosphorylation in prokaryotic cells. In this primary study we report the occurrence and characterization of phosphotyrosine (P-Tyr) proteins, as well as protein tyrosine kinase and protein tyrosine phosphatase (PTPase) activities, in *Anabaena* sp. strain PCC 7120, a cyanobacterium for which a range of tools for genetic manipulation has been developed (9, 32).

MATERIALS AND METHODS

Strains and plasmids. A clonal axenic culture of *Anabaena* sp. strain PCC 7120 was obtained from Jeff Elhai, University of Miami, Miami, Fla. *Anabaena* sp. strain PCC 7120 was grown at 28°C in BG-11 or BG-11₀ medium (22) under a photon flux density of approximately 500 μmol of photons $\text{m}^{-2} \text{s}^{-1}$ with a 12-h light–12-h dark cycle. Modifications to these growth conditions are described in the figure legends. Plasmid pSK-PTP1B was obtained from N. Tonks and A. J. Flint (Cold Spring Harbor Laboratory) and was maintained in *Escherichia coli* BL21(DE3). Plasmid pRSET-PTP1B was obtained from K. Bischoff.

Protein and enzyme purification. Cells were harvested by centrifugation and dispersed in a ratio of 1:1 with lysis buffer. Lysis buffer contained 50 mM HEPES (pH 7.5), 20 mM MgCl₂, 20 mM KCl, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM β -mercaptoethanol, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 0.1% (wt/vol) Tween 20, 1 μM leupeptin, 0.07 μM benzamide, 0.01% (wt/vol) thimerosol, 25 μM diisopropyl fluorophosphate, and 50 μM phenylmethylsulfonyl fluoride. To disrupt the cells, the suspension was either passed through a chilled French pressure cell at 110 MPa or ground with alumina powder (type A5, Sigma no. A-2039) in a chilled mortar.

Recombinant IphP was purified from *E. coli* KM001 cells as described elsewhere (21). The gene encoding human placental PTPase 1B (PTP-1B) was cloned in pRSET A (Invitrogen, San Diego, Calif.) as pRSET-PTP1B and expressed in *E. coli* BL21(DE3). Lysates of the latter cells in a mixture of 20 mM Tris-HCl (pH 7.2), 5 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 0.25 mM sucrose were applied to a 2-ml column packed with Ni²⁺-charged Sepharose (Pharmacia LKB, Piscataway, N.J.). The column was washed first with 20 mM Tris-HCl (pH 7.2), 500 mM NaCl, and 5 mM imidazole, and then His-tagged PTP-1B was eluted with 250 mM imidazole in 20 mM Tris-HCl (pH 7.2) and 500 mM NaCl.

Western blotting and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). All Western analyses reported here employed RC20H recombinant monoclonal antibodies (Transduction Laboratories, Lexington, Ky.). The

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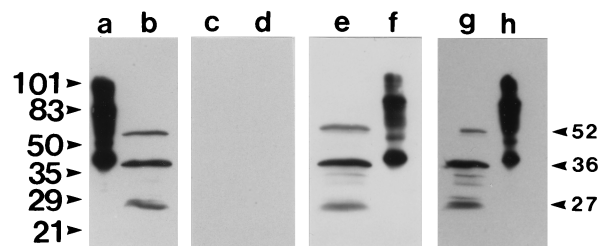


FIG. 1. *Anabaena* sp. strain PCC 7120 contains three major proteins that are phosphorylated on tyrosine. Cells were grown in BG11₀ medium to late stationary phase, at room temperature, with an incident photon flux density of approximately 100 μmol of photons $\text{m}^{-2} \text{s}^{-1}$. Cell extracts were transferred to SDS-15% (wt/vol) PAGE gels (approximately 20 μg of total protein per lane in lanes b, d, e, and g). Lanes a, c, f and h were loaded with 2 μl of a lysate of A431 human epidermoid carcinoma cell line that contained tyrosine-phosphorylated epidermal growth factor receptor (Transduction Laboratories). Western blots were prepared with RC20H antibodies (lanes a and b) or RC20H antibodies preincubated in the presence of either 5 mM P-Tyr (lanes c and d), 5 mM phosphoserine (lanes e and f), or 5 mM phosphothreonine (lanes g and h). Arrowheads denote the positions of molecular mass markers in kilodaltons.

antibodies are covalently linked to horseradish peroxidase, and an enhanced chemiluminescence system (Supersignal Substrate; Pierce, Rockford, Ill.) was used for their detection. General conditions for electrophoresis and immunoblotting were as described previously (21) with the exception that the RC20H antibodies were used at a dilution of 1:2,500 and immunoblots were blocked for 1 h in TBST buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05 Tween 20) with 1% (wt/vol) bovine serum albumin (Sigma no. A7906) prewarmed at 37°C.

Dephosphorylation of tyrosine-phosphorylated proteins. Cell extracts of *Anabaena* sp. strain PCC 7120 with total protein concentrations of approximately 20 mg ml^{-1} were incubated in 50 mM HEPES (pH 7.0) at 30°C for 2 h with either 1.5 mCi of [α - ^{32}P]ATP (0.2 mM; 10 nmol) or 1.5 mCi of [γ - ^{32}P]ATP (0.2 mM; 10 nmol). Proteins were resolved through SDS-PAGE and transferred to a polyvinylidene difluoride membrane (see above). Individual bands of protein were located on blots by autoradiography prior to excision. Portions of membrane fragments of equivalent surface area were incubated in the presence of either IphP (1 μg ; in 50 mM sodium acetate [pH 5.0]-2 mM DTT), PTP-1B (0.5 μg ; in 20 mM Tris-HCl [pH 7.0]-2 mM DTT-5 mM EDTA-0.25 M sucrose), or alkaline phosphatase (20 U; Sigma no. P-7923; in 50 mM Tris-HCl [pH 8.2]-2 mM DTT) or in each of the buffers alone (controls). Incubation was continued at 30°C overnight. The membrane fragments were removed, and levels of radioactivity both in supernatant fractions and in membrane fragments were determined through scintillation counting.

Phosphoamino acid analyses. ^{32}P -labeled proteins in cell extracts were transferred to polyvinylidene difluoride membranes through electroblotting. Membrane strips supporting protein bands were placed in 6 N HCl in glass vials that were sparged with nitrogen and then heated at 110°C for 1 h (10). The membrane fragments were dried under vacuum, rewetted with methanol, suspended in distilled water, and vortexed. The membrane was discarded, and the solution was taken to dryness. The residue was mixed with 5 μl of the pH 1.9 phosphoamino acid analysis buffer described by Cooper et al. (3) and spotted on a thin-layer chromatography plate together with standard phosphoamino acids. Conditions for two-dimensional electrophoresis and the detection of labeled and nonlabeled phosphoamino acids were as described elsewhere (3, 10).

PTPase assays. Cell extracts were assayed for in vitro PTPase activity by using dephosphorylated tyrosine-phosphorylated reduced carboxamidomethylated and maleylated lysozyme (RCML) as described previously (21) with the modifications noted in the figure legends. Additional assays were conducted at pH 5.0 (the pH optimum for RCML dephosphorylation by IphP from *N. commune* UTEX 584) and pH 7.0 (the pH optimum of the endogenous PTPase activity of *Anabaena* sp. strain PCC 7120).

RESULTS

***Anabaena* sp. strain PCC 7120 contains proteins that are phosphorylated on tyrosine in vivo.** Cell extracts of *Anabaena* sp. strain PCC 7120, grown under nitrogen-fixing conditions, contained three polypeptides of approximately 27, 36, and 52 kDa which cross-reacted strongly with P-Tyr-specific RC20H monoclonal antibodies (Fig. 1). Cross-reactions between these three proteins and RC20H antibodies were abolished completely when replicate blots were incubated in the presence of 5 mM P-Tyr. No such change in the immunoreactivity of the

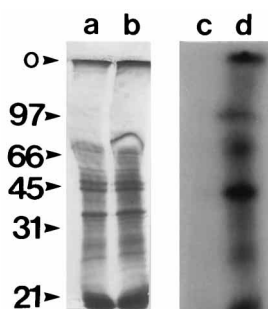


FIG. 2. *Anabaena* sp. strain PCC 7120 contains an activity that uses [γ - ^{32}P]ATP to phosphorylate proteins. Lysates were incubated in the presence of [α - ^{32}P]ATP (lanes a and c) or [γ - ^{32}P]ATP (lanes b and d) in 50 mM HEPES, pH 7.0, for 2 h and loaded (approximately 20 μg of total protein). Lanes a and b, a 12.5% (wt/vol) gel stained with Coomassie blue; lanes c and d, autoradiography. Molecular mass markers (in kilodaltons) are indicated with arrowheads. O, origin.

three polypeptides was observed if an equivalent concentration of either phosphoserine or phosphothreonine was used under the same conditions of incubation. A cell lysate of mouse RSV-3T3 cells (Transduction Laboratories) containing tyrosine-phosphorylated proteins served as a control in these experiments. Consistent results were obtained in three separate trials.

***Anabaena* sp. strain PCC 7120 contains P-Tyr kinase activity.** In preliminary trials to detect protein kinase activities, sodium orthovanadate and sodium fluoride were added to cell extracts to inhibit endogenous protein tyrosine and protein serine/threonine phosphatase activities, respectively. The extracts were then incubated at 25°C, with or without the addition of 1 mM ATP and 1 mM MgCl_2 , for different periods of time and were then resolved by Western blotting. For extracts that were incubated without addition of Mg-ATP, there was no obvious discernible change in the signal intensities attributed to the three major P-Tyr proteins. In contrast, the signal intensities associated with the three P-Tyr proteins increased according to the time of incubation of extracts after the addition of Mg-ATP (data not shown). More-detailed studies were

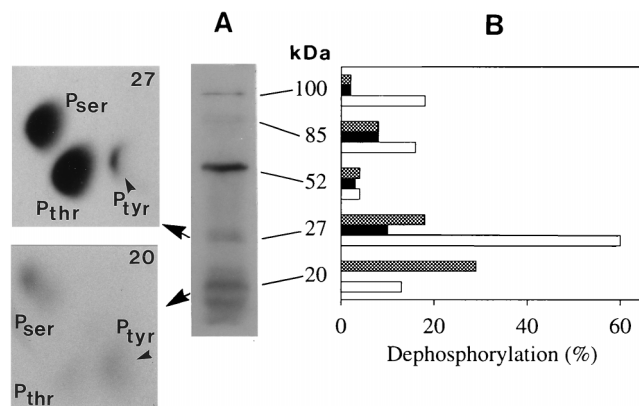


FIG. 3. Protein kinase activities in *Anabaena* sp. strain PCC 7120 phosphorylate proteins on P-Tyr, phosphoserine, and phosphothreonine. Cell lysates were incubated in the presence of [γ - ^{32}P]ATP as described in the legend to Fig. 2, and protein bands on blots were located through autoradiography (panel A, right lane). (B) Individual bands were incubated with either alkaline phosphatase (hatched), PTP-1B (black), or IphP (white) as described in Materials and Methods. (A) Two-dimensional chromatography and phosphoamino acid analysis of preparations of the 27- and 20-kDa proteins are shown (left panel).

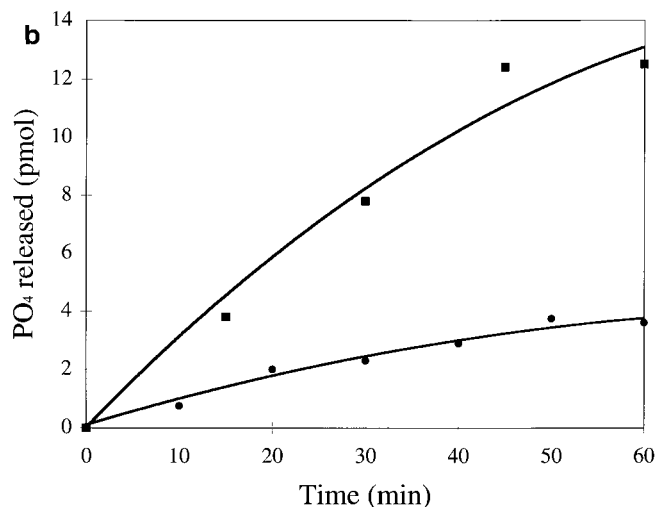
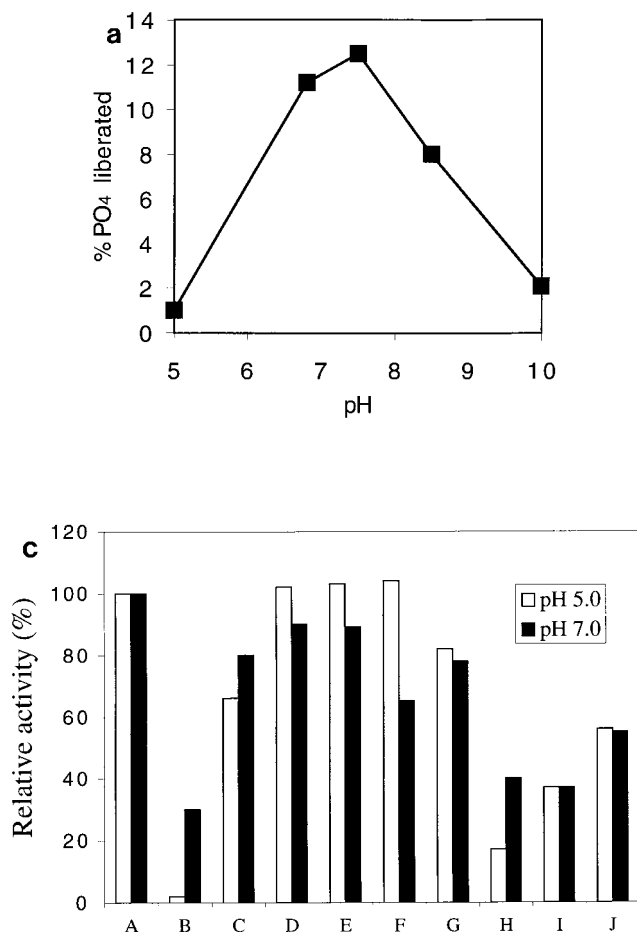


FIG. 4. *Anabaena* sp. strain PCC 7120 contains a PTPase activity. Extracts were prepared from cells grown under the conditions as described in the legend to Fig. 1. (a) PTPase activity was measured as the liberation of P_i from 2 μM RCML substrate in reaction volumes of 25 μl that included either 40 mM sodium acetate buffer (pH 5.0), 40 mM Tris-HCl (pH 7.0 or 7.5), or 40 mM sodium carbonate (pH 8.5 or 10.0) for 45 min. These different buffers (with different pK_a values) were used to determine activity over a broad range of pHs. (b) Time course of PTPase activity measured as described for panel a. Assays were conducted at pH 5.0 (●) and pH 7.0 (■). (c) The influence of effectors was measured for 30 min at 30°C in 25-μl reaction volumes which contained 5 μM RCML-P-Tyr substrate. A, no effector; B, 1 mM sodium orthovanadate; C, 10 mM sodium tartrate; D, 1 mM tetrakisole; E, 15 μM okadaic acid; F, 10 mM EDTA; G, 50 mM *N*-ethylmaleimide; H, 0.25 mg of heparin ml⁻¹; I, 10 mM *p*-hydroxymercuri-phenylsulfonic acid; J, 10 mM *p*-hydroxymercuribenzoate.

then conducted to detect protein tyrosine kinase activity. Incubation of cell extracts of *Anabaena* sp. strain PCC 7120 with [γ -³²P]ATP but not [α -³²P]ATP led to the phosphorylation of five polypeptides with molecular masses of 100, 85, 52, 27, and 20 kDa. Of these, the signals associated with the 52-, 27-, and 20-kDa polypeptides were the most prominent (Fig. 2). Two approaches were used to assess the form in which ³²P was bound to the different polypeptides. First, the presence of ³²P-Tyr, [³²P]phosphothreonine, and [³²P]phosphoserine was tested for by measuring the capacity of PTP-1B (P-Tyr specific) and IphP (dual specificity for P-Tyr and phosphoserine/threonine) to liberate ³²P from the labeled polypeptides (Fig. 3). All of the polypeptides except the 20-kDa form were dephosphorylated in the presence of PTP-1B, and all except the 20- and 50-kDa forms showed highest susceptibility to dephosphorylation in the presence of IphP. With the exception of the 20-kDa polypeptide, there was a marked consistency in the extents to which alkaline phosphatase and PTP-1B dephosphorylated labeled polypeptides. Both PTP-1B and IphP dephosphorylated the 27-kDa polypeptide to the greatest extent. Of the five proteins, the 52-kDa protein provided the strongest signal following autoradiography of blots, and presumably it contained greatest amounts of ³²P-phosphoamino acids (Fig. 3A). The 52-kDa protein showed least susceptibility to the effects of three different enzymes (Fig. 3B).

Not unexpectedly, there were some differences in the types of P-Tyr polypeptides detected with RC20H antibodies in vivo (Fig. 1) and those generated after incubation of extracts in

vitro with exogenous [γ -³²P]ATP (Fig. 3). For example, the appearance of a 20-kDa P-Tyr polypeptide after incubation under the latter conditions may reflect its nonspecific phosphorylation by the endogenous tyrosine kinase(s) and/or its release from a cell compartment which is inaccessible to the same kinase(s) in vivo. The ³²P-Tyr of this 20-kDa polypeptide (Fig. 3A) was presumably inaccessible after immobilization of the polypeptide since it was not released by PTP-1B (Fig. 3B).

The 27- and 20-kDa polypeptides were selected for comparative phosphoamino acid analysis. Both polypeptides contained ³²P-Tyr, [³²P]phosphoserine, and [³²P]phosphothreonine. The identification of ³²P-Tyr after phosphoamino acid analysis of the 27-kDa polypeptide is consistent with the dephosphorylation observed after incubation of the polypeptide with P-Tyr-specific PTP-1B (Fig. 3B). The presence of [³²P]phosphoserine and [³²P]phosphothreonine, in addition to ³²P-Tyr, accounts for the greater proportion of labeled phosphate liberated by the dually specific phosphatases IphP and alkaline phosphatase compared to those liberated by tyrosine-specific PTP-1B.

***Anabaena* sp. strain PCC 7120 contains P-Tyr phosphatase activity.** Cell extracts of *Anabaena* sp. strain PCC 7120 contained an intracellular activity (negligible activity was associated with the cell-free culture supernatant) that dephosphorylated tyrosine-phosphorylated RCML with a pH optimum around 7.5 (Fig. 4a and b). The activity was substantially inhibited in the presence of the PTPase inhibitor sodium orthovanadate (Fig. 4c). The sulfhydryl-modifying agents *p*-hydroxymercuri-phenylsulfonic acid and *p*-hydroxymercuribenzoate caused approximately 60 and 50% inhibition of the activity, respectively. The activity was also inhibited by heparin (11).

Regulation of P-Tyr protein synthesis and/or phosphorylation. An exhaustive analysis of the conditions that influence the

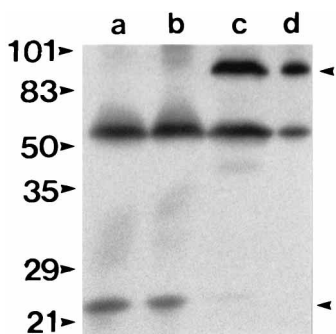


FIG. 5. Presence of P-Tyr proteins in *Anabaena* sp. strain PCC 7120 is influenced by light intensity. Western blot and analysis were as described for Fig. 1. Cells were grown in BG11 medium to stationary phase under 20 μmol of photons $\text{m}^{-2} \text{s}^{-1}$ (lanes a and b) or 300 μmol of photons $\text{m}^{-2} \text{s}^{-1}$ (lanes c and d). Extracts were obtained from cultures of different cell densities in independent experiments (experiment 1, lanes a and c; experiment 2, lanes b and d). Molecular mass markers (in kilodaltons) are indicated by arrowheads.

synthesis of P-Tyr proteins and their state of phosphorylation and dephosphorylation was beyond the scope of the present study. However, the 36-kDa P-Tyr protein which was detected in cells from cultures grown in the absence of combined nitrogen (Fig. 1) was not detected when cells were grown in the presence of combined nitrogen (Fig. 5). These data were obtained in multiple trials, with cultures of different ages and cell densities (Fig. 5). Cells grown under comparatively high photon flux densities, but not low photon flux densities, contained a 90-kDa P-Tyr protein which cross-reacted conspicuously with RC20H antibodies (Fig. 5). The approximately 27-kDa P-Tyr protein was detected only in cultures grown under low photon flux densities irrespective of the presence or absence of combined nitrogen (Fig. 1 and 5). Signals from all of these polypeptides were outcompeted in the presence of 5 mM P-Tyrosine.

DISCUSSION

The question of whether the members of the domain *Bacteria* covalently modify proteins via phosphorylation and dephosphorylation has been the source of considerable controversy (15). In this paper we report the detection of all three components of a protein P-Tyr modification system in the cyanobacterium *Anabaena* sp. strain PCC 7120: phosphotyrosyl proteins, protein tyrosine kinase activity, and PTPase activity. By using a monoclonal antibody to P-Tyr, three prominent polypeptides with molecular masses of ≈ 27 , 36, and 52 kDa were detected in cell extracts from *Anabaena* sp. strain PCC 7120. Several lines of evidence indicate that the antigen that elicited the immunoreaction was protein-bound P-Tyr. First, the antibody used was monoclonal. It is covalently linked to its reporter enzyme, horseradish peroxidase, and thus no secondary antibody was needed. This is crucial, since most commercially available secondary antibody preparations show strong, nonspecific immunoreactivity against bacterial proteins (18). Second, the immunoreaction was blocked completely and specifically through the addition of free P-Tyr but not phosphoserine or phosphothreonine. These controls are imperative for the assessment of the nature of signals from immunoanalyses. Third, two of these proteins (27 and 52 kDa) incorporated [^{32}P]phosphate when cell extracts were incubated with [γ - ^{32}P]ATP but not [α - ^{32}P]ATP. This pattern is consistent with a protein phosphorylation, as opposed to an adenylation, event. Fourth, after blotting to a membrane, a significant portion of the [^{32}P]phosphate in these proteins was removed following

incubation with PTP-1B. Since this enzyme is known to be specific for P-Tyr (12, 28), these data confirm that a significant portion of the protein-bound phosphate was in the form of P-Tyr. Fifth, subsequent phosphoamino acid analysis of two of the ^{32}P -phosphoproteins revealed the presence of phosphoserine and phosphothreonine as well as P-Tyr, a finding that is consistent with the fact that PTP-1B hydrolyzed only a fraction of the protein-bound radioactivity. Because phosphomonoester and peptide bond hydrolyses proceed simultaneously during phosphoamino acid analyses (see Materials and Methods), the data obtained for the two polypeptides should be viewed from a qualitative rather than a quantitative perspective. In fact, of the two polypeptides analyzed, a greater amount of ^{32}P , relative to the three ^{32}P -phosphoamino acids, was obtained after phosphoamino analysis of the 20-kDa polypeptide (data not shown).

Through these criteria we concluded that *Anabaena* sp. strain PCC 7120 contained tyrosine-phosphorylated proteins. They must therefore also harbor protein tyrosine kinase(s), and the observation that part of the phosphate incorporated into proteins when cell extracts were incubated with [γ - ^{32}P]ATP was in the form of ^{32}P -Tyr confirmed this. Using an exogenous phosphotyrosyl protein, RCML lysozyme (21), we then asked whether *Anabaena* sp. strain PCC 7120 extracts also contained protein tyrosine phosphatase activity in addition to protein tyrosine kinase activity. The answer was affirmative. Several lines of evidence suggest that the activity appeared to derive from a PTPase and not from a nonspecific alkaline or acid phosphatase. First, the pH optimum of the enzyme was in the neutral range. Second, the activity could be detected only following cell rupture. No PTPase activity could be detected in the cell growth media, even after it had been concentrated manifold. Third, the activity was inhibited by orthovanadate and sulfhydryl modifying reagents, both of which are known to inhibit the activity of well-characterized PTPases (20, 28).

The principal focus of this investigation was to identify the components of a protein tyrosine phosphorylation network in *Anabaena* sp. strain PCC 7120. During the course of the study, however, preliminary data which suggest at least two potential forms of regulation of this protein modification were obtained. First, the phosphorylation status and/or abundance of at least two P-Tyr proteins in *Anabaena* sp. strain PCC 7120 changed markedly depending on the photon flux density to which the cells were subjected. Second, the patterns of P-Tyr proteins detected in cultures grown in the presence or absence of combined nitrogen were quite different (Fig. 1 and 5).

Previous studies suggested that protein phosphorylation and two-component regulatory systems play an important role in physiological and developmental responses of cyanobacteria, including their responses to osmotic shock, high light, ammonia, chromatic adaptation, and the presence of intermediary metabolites at physiological concentrations (7, 8, 16, 23, 25, 31, 34). Although two-component regulatory systems appear to be involved in several specific aspects of cyanobacterial physiology (17), we consider it probable that protein tyrosine phosphorylation, in particular, plays a central role in the overall integration of metabolism, as it does in eukaryotic cells (15). The first evidence of the potential importance of tyrosine phosphorylation in cyanobacteria was obtained upon biochemical characterization of the dual-specificity protein tyrosine serine/threonine phosphatase IphP from *N. commune* UTEX 584 (21). The data presented here provide evidence for all components of a tyrosine phosphorylation signal transduction network in *Anabaena* sp. strain PCC 7120. Because this strain is amenable to genetic manipulation, it will be possible to explore the role of this protein modification event and its consequences in detail.

ACKNOWLEDGMENTS

This work was supported by grants from the NSF (IBN 9513157 to M.P.) and the NIH (GM45368 to P.J.K.).

We thank Ken Bischoff for construction of the plasmid that encodes His-tagged PTP-1B and its purification. We are grateful to T. J. Larson for reading critically the manuscript.

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