Regulation of protein phosphorylation in the cyanobacterium *Anabaena* strain PCC 7120

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Protein kinase activities have been detected in cell-free extracts of the cyanobacterium *Anabaena* PCC 7120. At least 12 polypeptides in the soluble fraction were phosphorylated *in vitro* at the expense of [γ - 32 P]ATP and the pattern of phosphorylation was shown to be regulated by intermediary metabolites and other effectors, at physiological concentrations. Glucose 6-phosphate exerted a regulatory effect on a phosphopolypeptide of M_r 56000 (p56) by stimulating a protein phosphatase, whereas ribulose 5-phosphate inhibited the corresponding protein kinase. In addition, DTT and the calmodulin antagonist trifluoperazine influenced the phosphorylation state of several different polypeptides, indicative of control by redox conditions and a calmodulin-like mediator, respectively. Furthermore, it was established that the phosphorylation of p56 required Mg^{2+} ($> 100 \mu M$) whereas that of a polypeptide of M_r 16000 occurred in the absence of Mg^{2+} and was inhibited by high concentrations ($> 1 mM$) of this cation. Several of the phosphopolypeptides detected *in vitro* corresponded in mobility on SDS-PAGE to species phosphorylated *in vivo*.

Introduction

It is becoming increasingly clear that protein phosphorylation plays an important role in the control of bacterial metabolism (see Cozzzone, 1988; Stock *et al.*, 1989). This form of post-translational modification of proteins provides a mechanism by which organisms can respond rapidly to changes in their internal and external environments over timescales which would preclude a transcriptional response. Protein phosphorylation may also provide a mechanism by which transcription may be modulated in response to such environmental alterations. Protein phosphorylation may involve three different types of covalent bonds with phosphate groups. The hydroxylated amino acids produce monoesters, whereas basic and acidic amino acids form phosphoramidates and acyl phosphates, respectively. Initially, studies on protein phosphorylation in bacteria tended to concentrate on proteins which were phosphorylated via a monoester linkage since it was thought that proteins containing phosphoramidates or acyl phosphates might

represent enzymic intermediates. However, it has been recently established that there is a highly conserved and widely distributed family of proteins which constitute two-component systems involved in signal transduction and that phosphoramidates and acyl phosphates are the phosphate linkages found in these systems (see Stock *et al.*, 1989).

In cyanobacteria, the primary nutritional mode is photoautotrophy, though many species are capable of photoheterotrophic growth at the expense of organic substrates such as glucose or fructose, and some are also capable of chemoheterotrophic growth at the expense of these carbon sources (see Smith, 1982). In addition, when cyanobacteria are transferred from photoautotrophic conditions into the dark they utilize glucose, derived from storage polysaccharides, via the oxidative pentose phosphate pathway (see Smith, 1982). It is likely that such metabolic transitions involve rapid modulation of some enzyme activities via post-translational mechanisms prior to a more general (and slower) transcriptional response. Although it has been well established that a number of enzymes involved in carbon, nitrogen and sulphur metabolism in cyanobacteria are subject to regulation by thioredoxins, and may be activated or inactivated depending on the redox state of these modulators (see Rowell *et al.*, 1988; Crawford *et al.*,

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Abbreviations: Fru-6-P, fructose 6-phosphate; Fru-1,6-P₂, fructose 1,6-bisphosphate; Glc-6-P, glucose 6-phosphate; Ru-5-P, ribulose 5-phosphate.

1988), it is possible that the activity of other enzymes may be controlled by protein phosphorylation. Protein phosphorylation has been demonstrated in only two cyanobacterial species, *Fremyella diplosiphon* PCC 7601 (Schuster *et al.*, 1984) and *Synechococcus* PCC 6301 (Allen *et al.*, 1985; Sanders *et al.*, 1989) and is thought to play a regulatory role in the chromatic acclimation (photosystem stoichiometry adjustment) of *Synechococcus* PCC 6301 in response to changes in the spectral quality of light.

One approach to the study of protein phosphorylation is to analyse protein kinase activities in cell-free extracts in conjunction with analyses of the resulting phosphopolypeptide profiles on SDS-PAGE. The addition of key metabolites or other potential effectors to such *in vitro* assays may reveal differential enhancement or inhibition of phosphorylation among specific polypeptide species (Londesborough, 1986). Therefore, *in vitro* kinase studies may help to recognize metabolic pathways in which protein phosphorylation is involved as a regulatory mechanism and to ultimately identify and characterize the individual components comprising such systems. It is important, however, to establish that the patterns of phosphorylation *in vitro* resemble those obtained *in vivo*. We have used both approaches to analyse protein phosphorylation in the filamentous heterocystous N_2 -fixing cyanobacterium *Anabaena* PCC 7120, as a preliminary step in establishing the role of protein phosphorylation as a regulatory mechanism in this photoautotrophic organism.

Methods

Organism and growth. The strain under study was *Anabaena* PCC 7120 (Rippka *et al.*, 1979) which, according to DNA/DNA hybridization studies by Lachance (1981), should be assigned to the genus *Nostoc*. This strain is available from the Culture Collection of Cyanobacteria at the Institut Pasteur, Paris, France.

For the phosphorylation studies, a single-colony isolate (WTg1) was made from a stock culture of *Anabaena* PCC 7120 to ensure homogeneity of the cell population.

For the preparation of extracts, Erlenmeyer flasks (1 l) containing 500 ml of medium BG11₀ (Rippka *et al.*, 1979) and filter-sterilized $NaHCO_3$ (10 mM) were inoculated to an initial OD_{750} of 0.03 (measured in a Kontron Uvikon model 860 spectrophotometer). If desired, this medium was supplemented with glucose (50 mM), $NaNO_3$ (10 mM) or both. Glucose had no effect on the growth rate under light-saturating conditions but slightly enhanced the growth yield once light became limiting. The cultures were agitated with magnetic stirring bars and were gassed with air/ CO_2 (99:1, v/v) introduced above the surface of the culture via cotton plugs equipped with sterile Pasteur pipettes (Rippka *et al.*, 1981). Light was supplied continuously by a ramp of three fluorescent lamps (Mazdafluor, Blanc Industrie) providing a photon flux density of $50 \mu mol m^{-2} s^{-1}$ at the surface of the culture vessels. Under these conditions the doubling times of the cultures during exponential growth (up to an OD_{750} of 0.7–0.8) were about 20–24 h, irrespective of the medium employed.

The purity of the cultures was verified as described by Rippka *et al.* (1979).

Cultures were harvested by centrifugation (20 min, 10000 g, 4 °C) at the end of exponential growth (at an OD_{750} of 0.8–1.2, equivalent to a total protein content of 120–180 $\mu g ml^{-1}$) and the volumes were adjusted to give pellets of 15–20 mg total protein (determined by the Lowry method). After one wash (20 ml) in the buffer of choice [50 mM-HEPES/NaOH, pH 7.5, containing 20 mM- $MgCl_2$ (HM buffer) or 50 mM Tris/maleate, pH 7.5 (TM buffer)], the pellets were either frozen at -70 °C or used immediately for the preparation of extracts.

Preparation of extracts. All steps in the preparation of extracts, including harvesting of the cells, were performed at 4 °C, and the buffers, glassware and other equipment were sterilized by filtration or by autoclaving, as appropriate.

The cell pellets (see above) were resuspended in HM or TM buffer (5 ml) and transferred into Corex centrifuge tubes (15 ml) containing 3 g sterile glass beads (0.11 mm diameter, Braun). The tubes were closed with rubber sleeves and were vortexed for 4–12 min (see below) on a vortex mixer (Cenco Instrumenten, Breda, The Netherlands) at maximum speed with cycles of 2 min and intermittent cooling (2 min) on ice. The beads were then allowed to settle and the broken cell suspensions were transferred to clean Corex tubes. The beads were washed with 1 ml of the appropriate buffer and the washing suspension pooled with the corresponding broken cell suspension.

The degree of cell breakage was estimated by microscopic examination of these crude extracts, in conjunction with measurements of the phycobiliprotein content of the supernatants produced after precipitation of membrane fragments with streptomycin sulphate (Tandeau de Marsac & Houmard, 1988). The short (4 min) glass bead treatments used for the experiments shown in Figs 1(a) and 1(b) were sufficient to break about 75% of the vegetative cells but did not result in a significant degree of heterocyst breakage. In order to ensure efficient breakage of both cell types, the glass bead treatment was extended to a total of 8–12 min for the experiments shown in Figs 2–4.

The soluble fractions of extracts (5 ml) were obtained by two successive centrifugations (15 min, 1000 g followed by 30 min, 6000 g). For particulate fractions the pellets of the 6000 g centrifugation were washed in the same volume (5 ml) of fresh buffer and recentrifuged (30 min, 6000 g) before being finally resuspended in 2.5 ml of buffer containing 0.1% Triton X-100.

For comparative analyses of extracts from cells grown under different culture conditions the protein contents were normalized by dilution after protein determination by the method of Bradford (1976) using the Bio-Rad protein assay reagent.

Protein kinase assay. Samples (10 μl , containing 10–20 μg protein) of cell-free extracts were incubated at room temperature with 185 kBq (5 μCi) of $[\gamma\text{-}^{32}P]ATP$ (Amersham, 110 TBq $mmol^{-1}$), appropriately diluted in sterile H_2O , in a total reaction volume of 20 μl and at a final concentration of 83 nM. The addition of unlabelled ATP (up to 10 μM) altered neither the pattern of protein phosphorylation nor the intensity of labelling. Since preliminary time-course experiments indicated that the kinase reactions were linear over the course of 1 h, all experiments (unless otherwise stated) were performed for this period in the absence of added unlabelled ATP. Intermediary metabolites and other effectors were dissolved in H_2O and added to the kinase reaction at a final concentration of 1 mM, unless otherwise stated. Reactions were terminated by the addition of an equal volume of double-strength denaturation buffer (Laemmli, 1970) and boiling for 4 min. Membrane fractions assayed for kinase activity were centrifuged (2 min, 12000 g) after denaturation to remove insoluble material. The entire sample was loaded onto the gel for analysis by SDS-PAGE.

In vivo labelling. For *in vivo* labelling with ^{32}P orthophosphate, the growth conditions were similar to those described for the preparation of extracts, except that the culture volumes were reduced to 50 ml, contained in Erlenmeyer flasks (150 ml) fitted with side arms. Light

was provided by a ramp of three fluorescent tubes (Claude 13W/B1) yielding a photon flux density of $50\text{--}60\ \mu\text{mol m}^{-2}\text{ s}^{-1}$ at the surface of the culture vessels. Growth was monitored with a Corning colorimeter (580 nm filter) allowing measurements directly on the side arms of the vessels. From the OD_{750} (Kontron)/ OD_{580} (Corning) ratio, the protein content of the cultures was estimated on the basis of the relationship: OD_{750} of 1 $\equiv$ $150\ \mu\text{g protein ml}^{-1}$. At an OD_{750} of 0.2–0.3 the cultures were supplemented with [^{32}P]orthophosphate (Amersham, 370 MBq ml^{-1} , acid-free, carrier-free) to a final activity of 740 kBq ($20\ \mu\text{Ci}$) ml^{-1} by means of a sterile disposable syringe. After further incubation for 24 h, the cultures were harvested (15 min, 1000 g), washed in 6 ml HEPES/NaOH buffer (50 mM, pH 7.5) and recentrifuged (5 min, 1000 g). The resulting pellets were resuspended in 1.5 ml of the same buffer and half of this cell suspension was frozen ($-20\ ^\circ\text{C}$) in Correx tubes (15 ml) with no further treatment. The remainder was stored as a pellet at $-20\ ^\circ\text{C}$ after centrifugation (2 min, 12000 g) in an Eppendorf microfuge. The total protein content of each sample was 2–2.5 mg.

Samples stored as pellets were resuspended in 800 μl single-strength denaturation buffer (Laemmli, 1970) and heated for 4 min at $100\ ^\circ\text{C}$. Cell debris and other insoluble materials were removed by centrifugation in an Eppendorf microfuge (2 min, 12000 g) and the supernatants were refrozen ($-20\ ^\circ\text{C}$). Samples stored as cell suspensions (750 μl) in Correx tubes were supplemented with 1.75 ml HEPES/NaOH buffer (50 mM, pH 7.5) and 1.5 g sterile glass beads. After 4×2 min breakage by vortexing (see above), MgCl_2 was added to a final concentration of 1 mM together with DNAase I and RNAase A, both at a final concentration of $10\ \mu\text{g ml}^{-1}$. After incubation (30 min at room temperature) the extracts were centrifuged (5 min, 1000 g) to remove large cell debris and the supernatants were frozen ($-20\ ^\circ\text{C}$) as small samples (500 μl) until required.

For SDS-PAGE the extracts (20 μl) prepared by glass bead treatment were incubated at $100\ ^\circ\text{C}$ for 4 min after addition of an equal volume of double-strength denaturation buffer (Laemmli, 1970) and the entire samples (containing equal protein loadings of about 20 μg) were applied onto the gel.

SDS-PAGE and autoradiography. Exponential gradient polyacrylamide (10–30%, w/v) gels were prepared and run as described by Turner & Mann (1986) and silver stained by the method of Wray *et al.* (1981) prior to drying and autoradiography at $-70\ ^\circ\text{C}$ with a Dupont Cronex Lightning Plus intensifying screen. The pattern of labelled polypeptides was highly reproducible for control samples, although the migration and resolution varied slightly in different experiments. Metabolites and other compounds that significantly influenced the pattern of phosphorylation were tested for their effects at least two or three times. SDS-PAGE gels containing samples which had been labelled *in vivo* were treated for 60 min at $78\ ^\circ\text{C}$ in 16% (w/v) trichloroacetic acid, washed as described by Manai & Cozzzone (1982) and stained with Coomassie Brilliant Blue R250 prior to drying and autoradiography.

Phosphoamino acid analysis. Following *in vitro* labelling of proteins and subsequent SDS-PAGE the polypeptides were transferred to an Immobilon-P membrane (Millipore) by Western blotting. The membrane was air dried and subjected to autoradiography. Regions of the membrane corresponding to labelled polypeptides of interest were cut out and incubated in Eppendorf tubes with 300 μl HCl (6 M) at $110\ ^\circ\text{C}$ for 2 h. The membrane was then removed from the tube and the remaining hydrolysate was freeze-dried. Phosphoamino acids in the protein hydrolysate were then identified as described by Turner & Mann (1986) using the *O*-phosphorylated derivatives of serine, threonine and tyrosine as standards.

Activity of glucose-6-phosphate dehydrogenase (EC 1.1.1.49). Extracts were prepared in TM buffer and assayed for Glc-6-P dehydrogenase activity as described by Schaeffer & Stanier (1978).

Chemicals. All intermediary metabolites employed, together with ATP, cAMP, cGMP, dithiothreitol, trifluoperazine, HEPES, streptomycin sulphate, deoxyribonuclease I, ribonuclease A and phosphoamino acids were obtained from Sigma. Glutathione (oxidized and reduced) was from Serva. Acrylamide and bisacrylamide (Electran grade) were from BDH and SDS from Serlabo. Protein standards for M_r estimations were obtained from Pharmacia. The protein assay reagent was obtained from Bio-Rad and radiochemicals from Amersham. All other chemicals were of the highest purity commercially available.

Results and Discussion

Influence of key intermediary metabolites and other effectors on protein phosphorylation in vitro

To establish whether protein kinase activities were detectable *in vitro*, a cell-free extract of *Anabaena* PCC 7120 was made in HM buffer from cells grown under N_2 -fixing conditions in the presence of glucose. The extract was separated into soluble and particulate fractions which were then assayed for protein kinase activities. Key intermediary metabolites and other potential effectors, all at concentrations of 1 mM, were added to establish whether they exerted any effect on the profile of phosphorylated polypeptides. It is clear that at least twelve polypeptides in the soluble fraction became phosphorylated under these conditions (Fig. 1*a*). Several of the effectors tested had a significant influence on the pattern.

DTT stimulated the phosphorylation of three polypeptides of M_r 83000, 78000 and 34000 (Fig. 1*a*). It seems likely that this modulation of kinase/phosphatase activities in response to DTT could be mediated via thioredoxins, which have been demonstrated to control the activities of a variety of enzymes in cyanobacteria (see Rowell *et al.*, 1988; Crawford *et al.*, 1988).

Fructose 6-phosphate (Fru-6-P) and fructose 1,6-bisphosphate (Fru-1,6-P₂) markedly reduced the incorporation of label into a polypeptide of M_r 56000 (p56) while having no effect on the incorporation into other phosphopolypeptides (Fig. 1*a*). A similar experiment (Fig. 1*b*) with additional effectors showed that glucose 6-phosphate (Glc-6-P) and ribulose 5-phosphate (Ru-5-P) also markedly reduced incorporation into the p56 polypeptide. Protein phosphorylation was not affected by cyclic nucleotides, in agreement with observations made with other bacteria (Skorko, 1984; Dadssi & Cozzzone, 1985; Holuigue *et al.*, 1985; Londesborough, 1986).

The differential action of trifluoperazine (TFP), which inhibited phosphorylation of the M_r 43000 and 30000 polypeptides, stimulated phosphorylation of a polypeptide of M_r 18000 but did not affect other species (Fig. 1*b*), is particularly interesting. TFP is a member of the

Fig. 1. Effects of intermediary metabolites and other effectors on the phosphopolypeptide patterns of *Anabaena* PCC 7120, grown under N_2 -fixing conditions in the presence of glucose and labelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ *in vitro*. The protein kinase reactions were performed on high-speed supernatants (10 μg protein per assay) of cells disrupted for 4 min with glass beads in HM buffer (see Methods). Additions were as follows. (a): (1) control, no addition; (2) DTT; (3) potassium ferricyanide; (4) NAD; (5) NADH; (6) NADP; (7) NADPH; (8) ribulose 1,5-bisphosphate; (9) fructose 1-phosphate; (10) fructose 6-phosphate; (11) fructose 1,6-bisphosphate; (12) fructose 2,6-bisphosphate. (b): (1) control, no addition; (2) glucose 6-phosphate; (3) ribulose 5-phosphate; (4) 6-phosphogluconate; (5) cAMP; (6) cGMP; (7) reduced glutathione; (8) oxidized glutathione; (9) fructose; (10) glucose; (11) glutamine; (12) glutamate; (13) trifluoperazine; (14) NaHCO_3 . All additions were made to a final concentration of 1 mM except NaHCO_3 , which was used at 10 mM. The sizes ($10^{-3} \times M_r$) of the major phosphopolypeptides (prefixed 'p') and protein standards are shown on the left and right, respectively.

phenothiazines, which in eukaryotes have been shown to act as calmodulin antagonists (see Cheung, 1980). Relatively little is known about the role of calmodulins in prokaryotes, although a calmodulin-like protein has been demonstrated in *Escherichia coli* (Iwasa *et al.*, 1981) and the ability of TFP to inhibit specific physiological processes such as phosphate uptake by the cyanobacterium *Oscillatoria limnetica* has been taken as evidence for the occurrence and physiological significance of calmodulin in the latter photosynthetic prokaryote (Kerson *et al.*, 1984). Furthermore, Pettersson & Bergman (1989) have obtained biochemical and immunological evidence for the presence of calmodulin in three strains of *Anabaena* and Oñec *et al.* (1990) have reported a calcium-binding protein of M_r 18000 in *Nostoc* PCC 6720, with properties typical of the calmodulin family.

However, it must be recognized that phenothiazines may also exert a variety of effects on prokaryotic cells that do not necessarily involve a calmodulin-like molecule: Peach *et al.* (1986) have shown that phenothiazines inhibit light-dependent Ca^{2+} efflux via the $\text{Ca}^{2+}/\text{H}^{+}$ antiport in *Chlorobium vinosum* and Molnar *et al.* (1977)

demonstrated that these compounds can be used to select for mutants of *E. coli* which lack the Lon protease. If a calmodulin-like mediator were to be involved in the regulation of protein phosphorylation in *Anabaena* PCC 7120, then the observed inhibition of phosphorylation of the polypeptides p43 and p30 by TFP (which renders calmodulin inactive) *in vitro* may result from an inhibition of calmodulin-activated kinases, whereas the stimulation by TFP of phosphorylation of the polypeptide p18 could be due to the inactivation of a calmodulin-regulated protein phosphatase. In support of the hypothesis that a calmodulin-like molecule may interact with protein kinases or phosphatases in prokaryotes are the results of Londesborough (1986), who showed that brain calmodulin altered the pattern of *in vitro* phosphorylation in cell-free extracts of *Clostridium thermohydrosulfuricum*.

Analysis of protein kinase activity in the particulate fraction of cell extracts (Fig. 2) revealed the most pronounced incorporation of label into a polypeptide of M_r 29000 (p29). Since phosphorylation of this major phosphopolypeptide in the particulate fraction was inhibited by TFP (data not shown), as was that of a

Fig. 2. A comparison of polypeptides labelled *in vivo* with [32 P]orthophosphate and *in vitro* with [γ - 32 P]ATP. Lanes 1–4, phosphopolypeptides of extracts (20 μ g protein) prepared from cells labelled *in vivo*; lanes 5–12, phosphopolypeptides after *in vitro* labelling of the soluble fractions (lanes 5–8, 10 μ g protein) and the particulate fractions (lanes 9–12, 7 μ g protein) of extracts prepared from cells cultivated in the absence of radioactive phosphate. Growth conditions were as follows. Lanes 1, 5, 9, absence of combined nitrogen; lanes 2, 6, 10, absence of combined nitrogen but presence of glucose; lanes 3, 7, 11, presence of nitrate; lanes 4, 8, 12, presence of nitrate and glucose. The sizes ($10^{-3} \times M_r$) of the major phosphopolypeptides (prefixed 'p') and protein standards are shown on the left and right, respectively. For details of cell breakage and preparation of extracts, see Methods.

minor phosphopolypeptide (p30) of very similar mobility in the soluble fraction (see Fig. 1*b*), it is likely that these two polypeptides are identical, their detection in the soluble fraction being due to a slight degree of membrane contamination. In addition, two phosphopolypeptides (p43 and p46) identical in mobility and similar in degree of labelling to two polypeptides phosphorylated in the soluble fraction were detected, suggesting that these might be components of peripheral membrane proteins which tend to dissociate from the membranes during the extraction procedure. Except for TFP, which not only abolished phosphorylation of p29 but also inhibited that of the peripheral membrane polypeptides p43 and p46 (data not shown), none of the metabolites or other potential effectors tested (the same as those employed in the soluble fraction, see Fig. 1*a, b*) influenced the phosphopolypeptide pattern of the particulate fraction.

Comparison of polypeptides labelled *in vivo* and *in vitro*

In order to establish whether any of the polypeptides phosphorylated *in vitro* correspond to species phosphorylated *in vivo*, we compared the phosphopolypeptide patterns of extracts labelled *in vitro* at the expense of [γ - 32 P]ATP with those obtained from intact cells labelled for 1–2 generations with [32 P]orthophosphate *in vivo* (Fig. 2). Since growth conditions may influence the extent of protein phosphorylation both *in vivo* and *in vitro*, these

experiments were performed on cells and extracts derived from cultures grown in the presence and absence of combined nitrogen, with or without exogenous glucose. Preliminary experiments on samples labelled *in vivo* demonstrated that the phosphorylation patterns obtained upon extracting cell pellets directly in SDS sample buffer were similar to those obtained with cells disrupted with glass beads and treated with DNAase and RNAase prior to denaturation. However, the relatively high background due to contaminating nucleic acids was decreased by the latter treatment, which was therefore chosen for the comparative analysis shown in Fig. 2.

The results show clearly that a number of phosphopolypeptides labelled *in vivo* correspond in mobility on SDS-PAGE to those obtained with extracts labelled *in vitro* (Fig. 2). One of the more prominent phosphopolypeptides, p56, was more strongly labelled *in vivo* in cells grown photoautotrophically in the presence of nitrate than in those grown under N_2 -fixing conditions in either the presence or absence of glucose. However, the addition of glucose to cells growing in the presence of nitrate abolished this stimulation (Fig. 2). Interestingly, *in vitro* kinase assays of soluble fractions prepared from cells grown under equivalent conditions but in the absence of [32 P]orthophosphate gave the opposite pattern of phosphorylation of p56 to that observed *in vivo* (Fig. 2). These results suggest that: (1) the phosphorylation state of p56 *in vivo* is regulated by the supply of

combined nitrogen; (2) exogenous glucose may alter the control of phosphorylation exerted by the source of nitrogen; and (3) the degree of labelling of this polypeptide *in vitro* is inversely related to its phosphorylation state *in vivo* (i.e. low levels of *in vitro* protein phosphorylation are indicative of extensive *in vivo* phosphorylation). Although no attempt was made in this study to discriminate between protein phosphorylation in vegetative cells and heterocysts of *Anabaena* PCC 7120, p56 cannot be specifically associated with the heterocysts, since differentiation of this cell type is repressed in the presence of nitrate, irrespective of the presence or absence of glucose.

Labelling of the particulate fraction of extracts *in vitro* revealed that incorporation into p29 was not influenced by the growth conditions of the cells (Fig. 2, lanes 9–12) whereas some differences were observed for p46 and p43, assumed to be polypeptides loosely associated with membranes (see above). The presence of nitrate seemed to favour the association of p46 with the particulate fraction, whereas in extracts from cultures grown in the absence of nitrate this polypeptide partitioned predominantly with the soluble protein fraction. However, the sum of labelling of p46 in the two fractions seems to exclude major differences in the total degree of labelling of this polypeptide. In contrast, p43, occurring mainly in the soluble protein fraction, was more strongly labelled in extracts from cells grown in the absence than in the presence of nitrate, even after taking into account the slightly stronger incorporation into p43 in the particulate fraction prepared from cells grown in the presence of nitrate (Fig. 2, lanes 5–12).

Since the gel comparing *in vivo* and *in vitro* labelling was treated after electrophoresis with hot trichloroacetic acid to hydrolyse remaining nucleic acids, the acid-stable phosphopolypeptides (Fig. 2) must contain monoester linkages rather than phosphoramidates or acyl phosphates, whose bonds are labile under such conditions (Manai & Cozzone, 1982). This was confirmed for p56, whose phosphoamino acid was identified as phosphoserine (data not shown).

Attempts to correlate phosphorylation of polypeptide p56 with changes in Glc-6-P dehydrogenase activity

The M_r of about 56000 (Cossar *et al.*, 1984), and the regulatory effects of Glc-6-P and Ru-5-P on the *in vitro* phosphorylation of p56 (see above), suggested that p56 could possibly be a subunit of Glc-6-P dehydrogenase. Extracts were therefore prepared in TM buffer, optimal for measuring the activity of this enzyme (Schaeffer & Stanier, 1978). A comparison of the *in vitro* labelling of extracts prepared in HM or TM buffer showed only minor differences in the overall pattern and degree of

labelling, particularly if Mg^{2+} (10 mM) was added to the TM extracts (data not shown). However, examination of the polypeptide patterns of silver-stained gels revealed that the stability of some of the phycobilisome subunits was superior in TM buffer, and subsequent kinase experiments were performed in this buffer. Attempts to correlate Glc-6-P dehydrogenase activity with the phosphorylation state of p56 were unsuccessful: preincubation of TM extracts for 1 h with ATP at low concentration (100 μM , about 1000-fold higher than that promoting efficient phosphorylation of p56 in *in vitro* kinase reactions) did not alter the activity of this enzyme compared to controls preincubated in the absence of ATP. It seems unlikely that the inhibitory effect of ATP at higher concentrations (> 1 mM) on the activity of Glc-6-P dehydrogenase can be attributed to phosphorylation of the enzyme, since these concentrations of ATP were also inhibitory to the partially purified enzyme (Schaeffer & Stanier, 1978), which should have been free of the corresponding kinase. However, we cannot exclude the possibility that the kinase and substrate co-purify, as is often the case in mammalian and plant systems.

Effect of Mg^{2+} on protein phosphorylation

In order to establish the effect of Mg^{2+} on the pattern of protein phosphorylation, cell-free extracts in TM buffer were dialysed against the same buffer. Protein kinase activities were then assayed after the addition of $MgCl_2$ at different concentrations (Fig. 3). It is clear that the maximum incorporation of label into p56 required Mg^{2+} concentrations greater than 100 μM . However, the most striking observation on these dialysed extracts was the detection of a polypeptide (p16) of M_r 16000 whose phosphorylation was inhibited by high concentrations (> 1 mM) of Mg^{2+} . Since the presence of trace amounts of Mg^{2+} after dialysis could not be excluded, kinase reactions were also performed in the presence of EDTA (1–2 mM). Under these conditions p16 was strongly phosphorylated (data not shown), even in TM extracts not subjected to dialysis.

To determine whether high concentrations of Mg^{2+} exerted an effect on the phosphorylation of p16 by inhibiting the corresponding kinase or by activating a phosphatase, this cation (10 mM) was added either at the start of the kinase reaction or after 30 min of the 1 h incubation period. If Mg^{2+} at this concentration were to inhibit the kinase, one would expect that its addition after 30 min should lead to about 50% of incorporation of label compared to that observed in kinase reactions incubated for the total incubation period in the absence of Mg^{2+} . In contrast, if high concentrations of Mg^{2+} were to stimulate a phosphatase, its addition after 30 min

Fig. 3. Effect of Mg^{2+} concentration on protein phosphorylation *in vitro* of the soluble fraction (20 μ g protein) of extracts prepared in TM buffer from cells grown under N_2 -fixing conditions in the presence of glucose. The kinase reactions were performed on extracts extensively dialysed against the same buffer and after the addition of the following concentrations of $MgCl_2$: (1) no addition; (2) 10 mM; (3) 1 mM; (4) 0.1 mM. The last lane (5) shows the result of labelling for 30 min in the absence of Mg^{2+} , followed by further incubation (30 min) in the presence of Mg^{2+} (10 mM). The sizes ($10^{-3} \times M_r$) of the major phosphopolypeptides (prefixed 'p') and protein standards are shown on the left and right, respectively.

should lead to a much more pronounced decrease of incorporation of label. Since the latter was observed (Fig. 3, compare lanes 1 and 5), the results suggest that Mg^{2+} activates a phosphatase apparently specific for p16. This conclusion is based on two assumptions: (1) that incorporation was linear over the 1 h period and (2) that the phosphatase apparently specific for p16 has low activity at concentrations of Mg^{2+} below 1 mM and thus ^{32}P turnover is low. Although the linearity of the kinase reaction in these experiments was not directly examined by the addition of excess unlabelled ATP at different time intervals, the effect of Mg^{2+} on the phosphorylation of p56 indicates such a response: if Mg^{2+} (10 mM), required for efficient phosphorylation of p56, was added

at time zero of the kinase reaction the degree of labelling was about twice as strong as that observed following addition at the midpoint (30 min) of the reaction (Fig. 3, compare lanes 2 and 5), indicating that ATP could not have become limiting during the reaction. Furthermore, turnover of the phosphate group(s) in p16 at low concentrations of Mg^{2+} must be low or zero since the extensive net labelling of p16 observed under these conditions would otherwise be difficult to explain.

The phosphoamino acid of p16 was identified as phosphoserine (data not shown).

It should be noted that the proteins composed of the polypeptides p56 and p16, together with their kinases and phosphatases, are extremely stable: kinase reactions performed in TM extracts which were previously dialysed and stored at 0–4 °C for several weeks without addition of protease inhibitors showed unaltered phosphorylation properties (i.e. degree of labelling and response to regulation by Mg^{2+}) (data not shown).

Regulation of phosphorylation of polypeptide p56 by Glc-6-P and Ru-5-P

In order to determine whether Glc-6-P and Ru-5-P affected the phosphorylation of p56 (see Fig. 1b) by inhibiting a kinase or activating a phosphatase, these metabolites were added either individually or together 30 min after the start of the kinase reactions (which were performed for 1 h). The controls received either water, excess unlabelled ATP or unlabelled ATP in combination with one or both of the metabolites. Addition of an excess of unlabelled ATP to the control after 30 min of incubation led to only a slight reduction in the expected incorporation, which should be 50% of that observed after 1 h of incubation without added ATP (Fig. 4, compare lanes 1 and 5), indicating both linearity of the kinase reaction and low phosphatase activity.

In contrast, it is clear (Fig. 4) that Glc-6-P alone or in combination with Ru-5-P, both with and without unlabelled ATP, decreased the labelling of p56 by far more than 50%, suggesting that Glc-6-P stimulates the activity of a phosphatase, whereas the patterns of phosphorylation of p56 observed after addition of Ru-5-P alone or in combination with unlabelled ATP suggest that Ru-5-P inhibits a protein kinase.

Inorganic phosphate at concentrations of 10–50 mM stimulated phosphorylation of p56 and seemed to exert its effect by inhibiting the phosphatase (data not shown).

One of the major metabolic transitions for a cyanobacterial cell in the natural habitat is that from photoautotrophic growth in the light to heterotrophic respiratory metabolism in the dark. This is characterized by a rapid reduction in the activities of the enzymes of the Calvin cycle and a concomitant increase in the activities of the

Fig. 4. Differential effects of Glc-6-P and Ru-5-P on the phosphorylation state of p56. Samples (20 µg protein) of the soluble fraction of a cell-free extract (in TM buffer) prepared from cells grown under N_2 -fixing conditions in the presence of glucose were incubated with [γ - 32 P]ATP for 30 min prior to the addition of Glc-6-P (1 mM), Ru-5-P (1 mM) or ATP (500 µM), either individually or in combinations. The reactions were terminated after further incubation for 30 min. Additions were as follows: (1) H_2O , control; (2) Glc-6-P; (3) Ru-5-P; (4) Glc-6-P and Ru-5-P; (5) ATP; (6) Glc-6-P and ATP; (7) Ru-5-P and ATP; (8) Glc-6-P, Ru-5-P and ATP. The sizes ($10^{-3} \times M_r$) of the major phosphopolypeptides (prefixed 'p') and protein standards are shown on the left and right, respectively.

enzymes of the pentose phosphate pathway, permitting the oxidation of glucose from storage polysaccharides (see Smith, 1982). Phosphorylation of p56 was markedly inhibited by Fru-1,6- P_2 , Fru-6-P, Glc-6-P and Ru-5-P. This multiplicity of effectors can be simplified by considering their possible interconversion in cell-free extracts. It seems likely that both Fru-1,6- P_2 and Fru-6-P may exert their regulatory effect on the phosphorylation of p56 as the result of conversion to Glc-6-P via the activities of fructose-1,6-bisphosphatase and hexose-6-phosphate isomerase, which have been shown to be active in cyanobacterial cell-free extracts (see Smith, 1982). Glc-6-P, which stimulates the phosphatase of p56, is the substrate of Glc-6-P dehydrogenase, the first enzyme of the oxidative pentose phosphate pathway, and

decreases significantly after a shift from light to dark. In contrast, Ru-5-P, which inhibits the kinase of p56, is the product of the second enzyme of this pathway, 6-phosphogluconate dehydrogenase and, like 6-phosphogluconate (Pelroy & Bassham, 1972), may increase during such a transition.

Therefore the regulatory effects of Glc-6-P and Ru-5-P on the phosphorylation state of p56 *in vitro* could suggest that the enzyme activity associated with this protein may be primarily subject to regulation via a protein kinase/protein phosphatase system, during the transition from photoautotrophic growth in the light to respiratory metabolism in the dark. Differences in the phosphorylation state of p56 such as those observed *in vivo* (Fig. 2) during growth in the light may reflect the degree of accumulation of either of these two metabolites under different growth conditions.

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