Chlorosis Induced by Nutrient Deprivation in *Synechococcus* sp. Strain PCC 7942: Not All Bleaching Is the Same†

JACKIE L. COLLIER^{1,2*} AND ARTHUR R. GROSSMAN¹

Department of Plant Biology, Carnegie Institution of Washington, 290 Panama Street,¹ and Department of Biological Sciences, Stanford University,² Stanford, California 94305

Received 19 November 1991/Accepted 15 May 1992

Cell coloration changes from normal blue-green to yellow or yellow-green when the cyanobacterium *Synechococcus* sp. strain PCC 7942 is deprived of an essential nutrient. We found that this bleaching process (chlorosis) in cells deprived of sulfur (S) was similar to that in cells deprived of nitrogen (N), but that cells deprived of phosphorus (P) bleached differently. Cells divided once after N deprivation, twice after S deprivation, and four times after P deprivation. Chlorophyll (Chl) accumulation stopped almost immediately upon N or S deprivation but continued for several hours after P deprivation. There was no net Chl degradation during N, S, or P deprivation, although cellular Chl content decreased because cell division continued after Chl accumulation ceased. Levels of the light-harvesting phycobiliproteins declined dramatically in a rapid response to N or S deprivation, reflecting an ordered breakdown of the phycobilisomes (PBS). In contrast, P-deprived cultures continued to accumulate PBS for several hours. Whole PBS were not extensively degraded in P-deprived cells, although the PBS contents of P-deprived cells declined because of continued cell division after PBS accumulation ceased. Levels of mRNAs encoding PBS polypeptides declined by 90 to 95% in N- or S-deprived cells and by 80 to 85% in P-deprived cells. These changes in both the synthesis and stability of PBS resulted in a 90% decline in the PC/Chl ratio of N- or S-deprived cells and a 40% decline in the PC/Chl ratio of P-deprived cells. Therefore, although bleaching appears to be a general response to nutrient deprivation, it is not the same under all nutrient-limited conditions and is probably composed of independently controlled subprocesses.

The cyanobacterium *Synechococcus* sp. strain PCC 7942 (also known as *Anacystis nidulans* [R2] and very similar to *Synechococcus* sp. strain PCC 6301 [*Synechococcus leopoliensis*] [15]; these strain designations will be referred to together as *Synechococcus* spp.) is a unicellular, obligate photoautotroph requiring only inorganic nutrients and light for growth. Like other cyanobacteria, this organism exhibits a complex suite of morphological and physiological changes when deprived of an essential nutrient. One aspect of the nutrient deprivation response which appears to be general (i.e., occurs in response to limitation for any of a number of different nutrients) is the phenomenon of chlorosis, or bleaching. This change in cell appearance from normal blue-green to chlorotic yellow or yellow-green is largely due to changes in photosynthetic pigments.

The major pigments of *Synechococcus* spp. are chlorophyll *a* (Chl) and the phycobiliproteins allophycocyanin (APC) and phycocyanin (PC). These pigments are associated with the photosynthetic apparatus in the thylakoid membranes (for a review, see reference 8). Chl is an integral component of the photosynthetic reaction centers and their antennae. Phycobiliproteins are pigmented constituents of phycobilisomes (PBS), which are water-soluble light-harvesting complexes attached to the cytoplasmic sides of the thylakoid membranes (for detailed reviews of PBS structure, see references 8, 9, 13, and 14). The abundance of these pigments and other components of the photosynthetic apparatus varies with environmental conditions, such as light quality and quantity, as well as with nutrient supply.

There have been a number of studies documenting bleaching in *Synechococcus* spp. deprived of nitrogen (N), sulfur (S), phosphorus (P), carbon (C), or iron (Fe) (3, 16, 20, 21, 30, 37, 38, 41; for reviews, see references 2, 8, 9, and 35). Pigment abundances have been quantified both per cell and on a whole culture (e.g., protein or culture volume) basis. By both measures, phycobiliprotein (and PBS) content has been observed to decline in nutrient-deprived *Synechococcus* spp. Wanner et al. (41) reported that cellular Chl content also declines during both N and S deprivation, although Allen and Smith (3) and Ihlenfeldt and Gibson (20) observed no change in the Chl contents of N- and P-deprived cultures, respectively, and Schmidt et al. (37) observed a small increase in culture Chl content during S deprivation.

A few of these investigations have examined the molecular underpinnings of the bleaching process. Yamanaka and Glazer (46) demonstrated that the degradation of PBS in N-deprived *Synechococcus* sp. strain PCC 6301 occurs in an orderly manner, with the loss of the terminal PC hexamer and its associated linker occurring first. They observed that chloramphenicol prevents PBS degradation and concluded that the degrading activity is an unstable protein, as is also true in N-deprived *Synechocystis* sp. strain PCC 6803 (11). In N-deprived *A. nidulans* both PC and PC apoproteins are degraded and the synthesis of new PC is repressed (22).

Although it has been assumed that PBS degradation is a general response to nutrient deprivation (e.g., see references 7 and 35), PBS structure during bleaching in response to limitation for nutrients other than N has not been examined. Since P- and Fe-deprived cells maintain higher levels of PC (20, 33) than do N- or S-deprived cells, bleaching may not be the same under all conditions. In order to investigate this possibility and to facilitate the molecular analysis of mutants

* Corresponding author.

† Carnegie Institution of Washington Department of Plant Biology, publication number 1121.

which do not bleach properly (9a), we have undertaken a detailed characterization of changes in the photosynthetic pigments of *Synechococcus* sp. strain PCC 7942 during N, S, and P deprivation.

MATERIALS AND METHODS

Strain and culture conditions. *Synechococcus* sp. strain PCC 7942 was maintained on solid BG-11 medium (1) containing 1% Noble agar (United States Biochemical Corp., Cleveland, Ohio). The medium was modified by replacing ferric ammonium citrate with FeCl_3 (12 mg/liter), buffering the medium to pH 8.0 with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and providing the micronutrients Zn, Cu, and Co as chloride salts. Cultures were grown at 32°C in 50-ml culture tubes, bubbled with 3% CO_2 in air, and illuminated with approximately $50 \mu\text{E m}^{-2} \text{s}^{-1}$ from incandescent bulbs. Under these conditions the cells doubled every 6 h. BG-11 medium lacking nitrogen (–N), sulfur (–S), or phosphorus (–P) was prepared by replacing the NaNO_3 , MgSO_4 , or K_2PO_4 salts with an equimolar amount of NaCl, MgCl_2 , or KCl, respectively. The wash medium (–NSP) lacked N, S, and P.

Procedure for initiating nutrient deprivation. A 50-ml BG-11 liquid culture was inoculated from a plate stock. At approximately 1×10^8 cells/ml (mid-log-phase growth), the culture was diluted into multiple tubes to a density of 6.25×10^6 cells/ml. After 24 h of growth (to a density of approximately 1×10^8 cells/ml), each culture tube was harvested by centrifugation at $3,500 \times g$ for 5 min (25°C), resuspended in 20 ml of –NSP medium, harvested a second time, and resuspended in 1 ml of –NSP medium. At time zero, this concentrated suspension was diluted into 50 ml of experimental (–N, –S, or –P) medium. –N and –S media were inoculated to a density of 5×10^7 cells/ml (high density [17]), while –P medium was inoculated to a density of 5×10^6 cells/ml (low density [34]). Controls consisted of washed cells resuspended in BG-11 medium at both high and low densities. At predetermined times, 5 ml of a culture was removed for analysis of cell density and PC and Chl content. Samples (50 to 500 ml, as needed) of culture were harvested for RNA or PBS isolation.

PBS isolation and pigment and protein quantitation. Cell densities were determined by counting diluted samples in a hemocytometer at a magnification of $\times 400$. Whole-cell spectra from 350 to 800 nm were measured on a Beckman (Fullerton, Calif.) DU-70 spectrophotometer. The spectrophotometric response was not linear over the 10-fold range of cell densities analyzed during the first few hours of nutrient deprivation, which caused an overestimation of PC and Chl contents of low-density cultures. Measurement of Chl by acetone extraction (5) confirmed that this overestimation was an artifact. To avoid nonlinearity at later times, samples were diluted as required to keep the A_{800} between 0.1 and 0.3.

Chl levels were measured by an approximation from whole-cell absorbances by the following equation (modified from reference 5):

$$\text{Chl (milligrams per milliliter)} = 0.01497(A_{680} - A_{750}) - 0.000615(A_{620} - A_{750})$$

Values per cell were calculated by dividing by cell density.

PBS content was estimated by relying on quantification of PC, since virtually all of the PC is assembled into PBS (46). The loss of phycobiliprotein absorbance in samples of a

culture heated at 75°C for 8 min (48) was used to determine PC content according to the following equation:

$$[A_{620} - A_{750} (\text{unheated})] - [A_{620} - A_{750} (\text{heated})] = \text{PC per milliliter}$$

Although PC dominates the absorbance at 620 nm, APC and Chl also contribute. A_{750} was used as a correction for cell scattering, and values per cell were calculated by dividing by cell density. This method agreed with estimates obtained by using the equations of Arnon et al. (5) and was more sensitive to the low PC levels in N- or S-deprived cultures.

PBS were isolated by a modification of previously described procedures (4, 12). The post-Triton X-100 supernatant was layered onto a linear 0.25 to 1.5 M sucrose (in 0.65 M phosphate) gradient and centrifuged at $230,000 \times g$ for 3.5 h in a Vac50 rotor. Intact PBS were removed from the gradient and stored frozen. Concentrations of PC and APC in purified PBS were estimated spectrophotometrically from the following equations:

$$\begin{aligned} [\text{PC}] (\text{milligrams per milliliter}) &= (A_{620} - 0.71A_{650})/6.83 \\ [\text{APC}] (\text{milligrams per milliliter}) &= (A_{650} - 0.19A_{620})/5.99 \end{aligned}$$

These equations were generated from the absorption coefficients for an average of hexameric PC and APC subunits (14). Polypeptide components of the PBS were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (4) and visualized in the gel by staining with Coomassie blue G or R. Bands in wet gels were quantified on a Hoefer Scientific Instruments (San Francisco, Calif.) GS300 Transmittance/Reflectance scanning densitometer. Relative levels of various polypeptides were calculated by dividing the height of each peak (the area gave equivalent results) by the molecular weight of the polypeptide.

Intact PBS were fixed and stained for electron microscopic examination as described previously (4). A Philips EM 410 electron microscope was used to produce photomicrographs of the PBS. The numbers of rods per PBS and of PC hexamers per rod were determined for all intact, well-defined PBS in several micrographs. These data (see Fig. 5) indicated that the ratio of 27-kDa polypeptide to APC- α in nutrient-replete PBS should be 0.23 (4.6 27-kDa and 20 APC- α [14] polypeptides per PBS). The greater affinity of Coomassie dyes for linkers compared with for phycobiliproteins (i.e., basic vs. acidic proteins [29]) resulted in a 2.5-fold overestimation of this value by the densitometry technique (Table 1). Similar quantitative comparisons among the rod linkers themselves are difficult because the precise linker composition of rods containing more than three hexamers is unknown.

RNA isolation and Northern (RNA blot) analysis. RNA was isolated by two procedures. The first was a modification of the procedure of Laudenbach et al. (24), from which precipitation of nucleic acids after cell lysis was omitted. The lysate was adjusted to 0.4 g of CsCl per ml and layered directly over a 5.7 M CsCl cushion, and the RNA was pelleted at $150,000 \times g$ for 15 h. The second procedure was modified from that of Mohamed and Jansson (31). The aqueous phase after the first phenol extraction was added to 7.3 ml of water containing 3.2 g of CsCl and pelleted as described above. CsCl-purified RNA was solubilized in 0.5 ml TE (10 mM Tris, pH 8.0, 1 mM EDTA), extracted with chloroform, precipitated with ethanol, and finally resolubilized in water.

A 0.7-kbp *Pst*I-*Pvu*II fragment from plasmid pPC22 containing *cpcA2* (23) was used to quantitate the levels of PC

TABLE 1. Relative ratios of PBS components in PBS isolated from cells deprived of S, N, or P

Medium	Time	Estimated molar ratio (SD) ^a			
		PC/APC ^b	27-kDa linker/APC- α ^c	30-kDa/27-kDa linker ^c	33-kDa/27-kDa linker ^c
All	0	2.38 (0.11)	0.58 (0.11)	0.52 (0.1)	0.88 (0.1)
BG-11	24	2.75	0.57 (0.08)	0.69 (0.04)	0.97 (0.01)
BG-11	48	2.44	0.52 (0.12)	0.68 (0.01)	0.99 (0.06)
-S	12	1.33 (0.16)	0.57 (0.1)	0.12 (0.06)	0.68 (0.08)
-S	24	1.3 (0.08)	0.55 (0.18)	0 (0)	0.62 (0.1)
-S	48	1.25 (0.12)	0.35 (0.09)	0 (0)	0.65 (0.07)
-N	12	1.44 (0.09)	0.52 (0.08)	0.07 (0.05)	0.6 (0.09)
-N	24	1.2 (0.07)	0.39 (0.2)	0.02 (0.03)	0.49 (0.05)
-N	48	1.05 (0.03)	0.34 (0.07)	0 (0)	0.46 (0.13)
-P	12	2.28 (0.18)	0.38 (0.13)	0.33 (0.07)	0.89 (0.13)
-P	24	1.97 (0.35)	0.40 (0.09)	0.32 (0.11)	0.91 (0.1)
-P	48	2.01 (0.22)	0.51 (0.09)	0.29 (0.15)	0.86 (0.17)

^a Values are averages of at least two determinations each on at least two independent PBS preparations (except for the controls, which are from only one preparation). Values (not shown) were also determined for -S and -N at 2, 4, 6, 9, 18, and 36 h and for -P at 36 h.

^b Determined spectrophotometrically.

^c Determined by scanning densitometry of Coomassie-stained SDS-PAGE gels.

mRNA. A 2.0-kbp *HincII* fragment containing *apcA*, *apcB*, and *apcC* (19) was used to quantitate APC mRNA levels. The *rhd* gene probe was a 1.2-kbp *XhoI-SphI* fragment encoding the C-terminal half of the Rhd protein plus approximately 500 bp downstream of the translation termination site (24). The *phoA* gene probe was fragment C from Ray et al. (34). The 16S rRNA probe was a 1.9-kbp *XbaI* fragment from pAN4 (40) containing most of the 16S rRNA gene.

Electrophoresis of RNA in agarose gels, transfer of RNA to nitrocellulose paper, labeling of DNA fragments with random primers, and hybridizations (at 65°C) were performed as previously described (24, 36). Quantitation of hybridization signals was done with a Molecular Dynamics (Sunnyvale, Calif.) PhosphorImager. To standardize the quantitative data, blots were stripped by incubation in distilled water at 65°C and rehybridized with the 16S rRNA probe.

RESULTS

Growth curves from a representative experiment are shown in Fig. 1. After 48 h, cultures reached a stable state

FIG. 1. A representative experiment showing growth of *Synechococcus* sp. strain PCC 7942 in BG-11 at high (A, open circles) and low (B, open squares) density and in -S (A, open triangles), -N (A, open triangles), and -P (B, filled squares) media.

FIG. 2. Chl content per milliliter of culture (A and B) and per cell (C and D) for nutrient-replete and nutrient-deprived cells. Values in panels A and B are from the experiment shown in Fig. 1, and values in panels C and D are averages of at least three independent experiments, with error bars representing standard deviations and with the 100% value set to 2.24×10^{-14} g of Chl per cell. Symbols are as described in the legend to Fig. 1.

and remained virtually unchanged for at least a week thereafter. Cells washed and diluted into complete BG-11 medium at either density grew exponentially for 36 to 48 h (Fig. 1A, open circles, and 1B, open squares). N-deprived cells (Fig. 1A, open triangles) underwent a single slow division in the 6 to 12 h following nutrient removal, while S-deprived cells (Fig. 1A, closed circles) underwent two divisions, each with a normal generation time of about 6 h. P-deprived cultures (Fig. 1B, closed squares) appeared to delay cell division for the first 3 to 6 h following nutrient removal, after which they underwent four divisions with a normal generation time. The apparent delay in cell division may be an artifact of cell aggregation in P-free medium, making accurate cell counts difficult.

P deprivation was initiated at lower cell densities to ensure that cultures deprived of N, S, or P reached approximately the same final density, below the point of extensive self-shading, since bleaching may be inhibited in low-light conditions (e.g., high cell density [9a]). Bleaching in P-deprived cultures started at high cell densities and diluted during the course of the experiment was the same as bleaching in those started at low cell densities. Bleaching in response to N or S deprivation was also the same at either starting density.

Figures 2A and B show Chl per milliliter for the experiment presented in Fig. 1. Control cultures (Fig. 2A, open circles, and 2B, open squares) accumulated Chl exponentially. N-deprived cultures (Fig. 2A, open triangles) stopped accumulating Chl almost immediately. Chl accumulated slowly for only the first 6 h following S deprivation (Fig. 2A, closed circles). In P-deprived cultures (Fig. 2B, closed squares), Chl accumulation proceeded normally for the first 9 h, slowed between 12 and 24 h, and stopped after 24 h. Total Chl content of nutrient-deprived cultures remained relatively unchanged once accumulation ceased.

FIG. 3. PC content per milliliter of culture (A and B) and per cell (C and D) for nutrient-replete and nutrient-deprived cells. Values in panels A and B are from the experiment shown in Fig. 1, and values in panels C and D are averages of at least three independent experiments, with error bars representing standard deviations. Symbols are as described in the legend to Fig. 1.

Chl content per cell, averaged from three independent experiments, is shown in Fig. 2C and D. Chl content of nutrient-replete cells changed little over the course of the experiment (Fig. 2C, open circles, and 2D, open squares; the high values observed for low-density control cells at early time points were a spectrophotometric artifact [see Materials and Methods]). The Chl content of N-deprived cells (Fig. 2C, open triangles) dropped to about 50% of the initial value, and that of S-deprived cells (Fig. 2C, closed circles) dropped to 35% of the initial value. These reductions in Chl per cell could be accounted for by cell division. P-deprived cells (Fig. 2D, closed squares) appeared to have elevated Chl levels during the first 9 h, but by 24 to 36 h the Chl level dropped to about 35% of normal. The increase above low-density controls between 3 and 9 h after P deprivation is due to the apparent lag in cell division upon P deprivation. The decrease in Chl content of P-deprived cells observed between 12 and 24 h was probably due to dilution of existing Chl by cell division, as it was in N- and S-deprived cells.

Figures 3A and B show PC per milliliter from the same experiment shown in Fig. 1. Control cultures at both densities (Fig. 3A, open circles, and 3B, open squares) accumulated PC at an exponential rate. Both N- and S-deprived cultures lost PC in an almost linear fashion after inoculation into experimental medium (Fig. 3A, open triangles and closed circles, respectively). P-deprived cultures (Fig. 3B, closed squares) accumulated PC normally for the first 9 h and then more slowly until approximately 24 h, after which PC content remained steady.

Figures 3C and D show PC content per cell averaged from three experiments. Control cultures at both cell densities maintained a relatively constant amount of PC per cell during the course of the experiment (Fig. 3C, open circles, and 3D, open squares; the high initial values in low density control cells were a spectrophotometric artifact [see Mate-

rials and Methods]). During N and S deprivation (Fig. 3C, open triangles and closed circles, respectively), cellular PC content dropped rapidly for the first 12 h. PC per cell then declined more slowly throughout the experiment. The level of PC in P-deprived cells (Fig. 3D, closed squares) was elevated until 12 h after the initiation of P deprivation but declined to 20% of normal levels by 48 h. This pattern was similar to that observed for Chl content of P-deprived cells and resulted in a final PC/Chl ratio that was approximately 60% of that of nutrient-replete cells; these values are similar to values reported previously (20). In contrast, the PC/Chl ratio of N- and S-deprived cells was less than 10% of the normal ratio.

PBS purified from nutrient-deprived cultures were used to investigate PBS structure during bleaching. Spectrophotometric analysis (Table 1) showed that the PC/APC ratio of PBS isolated from N- or S-deprived cells declined linearly to approximately 50% of its initial value during the first 12 h. In contrast, PBS isolated from P-deprived cells showed only a 20% decrease in the PC/APC ratio.

A schematic diagram of PBS from *Synechococcus* sp. strain PCC 7942 is shown in Fig. 4A (13, 14). The major component of the core substructure is APC. Each PBS is usually represented as containing six rods, and each rod is usually represented as containing three PC hexamers, although PBS size varies in response to environmental parameters (26, 46). The core-proximal, middle, and terminal hexamers are associated with 27-, 33-, and 30-kDa linker polypeptides, respectively. Below the schematic is an SDS-polyacrylamide gel on which the polypeptides of PBS isolated at various times after S or P deprivation have been separated (Fig. 4B). During the first 18 h of S deprivation, the distal, 30-kDa linker was lost. Similar changes occurred in PBS from N-deprived cells, as has been reported previously (46). In contrast, PBS from P-deprived cells contained the 30-kDa linker throughout the experiment (Fig. 4B).

The structure of isolated PBS can be deduced by quantifying PBS components separated by SDS-PAGE (Table 1). The ratios of the rod linkers relative to one another provide a measure of rod length. In PBS from control cells, the 33-kDa/27-kDa and 30-kDa/27-kDa linker ratios suggested that most rods contained more than two hexamers. In PBS from both S- and N-deprived cells the 30-kDa/27-kDa linker ratio dropped to 0 during the first 18 h of nutrient deprivation. The 33-kDa/27-kDa linker ratio in PBS from S-deprived cells decreased 30%, and that from N-deprived cells declined 50%. In contrast, PBS from P-deprived cells exhibited only a 40% decrease in the 30-kDa/27-kDa linker ratio and no change in the 33-kDa/27-kDa linker ratio. The ratio of the 27-kDa rod linker to APC- α provides a measure of the number of rods per PBS. This ratio remained relatively constant in PBS from nutrient-replete cells, but decreased by 40% in PBS from cells deprived of N or S for 48 h. The 27-kDa linker/APC- α ratio declined temporarily during P deprivation but recovered later. These data suggest that PBS from cells deprived of N or S for 48 h lost half of their rods and that none of the remaining rods contained more than two hexamers. In contrast, the changes in PBS from P-deprived cells were much less severe, with only a portion of the terminal rod hexamers lost. Changes in PBS structure following N or S deprivation occurred after PBS synthesis had stopped, but those following P deprivation occurred while PBS were still being synthesized.

To confirm this structural analysis, the numbers of rods per PBS and of hexamers per rod were determined via electron microscopy of intact PBS from nutrient-replete cells

FIG. 4. (A) Schematic diagram of *Synechococcus* sp. strain PCC 7942 PBS, indicating positions of major PBS components. (B) Polypeptides of the PBS from cells deprived of S for up to 24 h (left panel) and deprived of P for 36 or 48 h (right panel) were separated by SDS-PAGE and stained with Coomassie brilliant blue. Approximately 10 μ g of APC were loaded per lane.

and from cells deprived of S for 48 h. Figure 5A shows the distribution of rod number per PBS (nutrient replete, solid; S deprived, cross-hatched). PBS from nutrient-replete cells had an average of 4.6 rods each. After 48 h of S deprivation, the average decreased by 30% (roughly comparable to the densitometric estimate) to 3.3 rods per PBS. The distribution of hexamers per rod is shown in Fig. 5B (nutrient replete, solid; S deprived, cross-hatched). The average rod length in PBS from nutrient-replete cells was 3.0 hexamers. After 48 h of S deprivation, the average rod length was reduced to 1.8 hexamers. Less than 10% of these rods contained more than two hexamers, as expected from the disappearance of the 30-kDa linker polypeptide, and 35% had fewer than two hexamers, as expected from the decrease in 33-kDa linker (Table 1). Together, the changes in rod length and in the number of rods attached to the core predicted a 50% decline in the PC/APC ratio (3.6 to 1.6), which agreed with the spectrophotometric estimation (Table 1).

To determine whether the reduction in PBS polypeptides

FIG. 5. Percent of the PBS population containing various numbers of rods (A) and of rods containing various numbers of hexamers (B) from nutrient-replete cells (solid bars) and from cells deprived of S for 48 h (cross-hatched bars). For nutrient-replete cells, 105 PBS containing 457 rods were counted. For cells deprived of S for 48 h, 78 PBS containing 250 rods were counted.

was reflected in the levels of mRNAs encoding those polypeptides, Northern (RNA blot) analyses were performed with RNA isolated from nutrient-deprived cells. Figure 6 shows autoradiograms of a Northern blot in which the RNA isolated from cells at various times after S deprivation was hybridized to four different labeled, gene-specific DNA fragments. The levels of PC and APC mRNAs reached minimum levels after 2.5 h of S deprivation (Fig. 6A and B). In contrast, an increased level of mRNA encoding Rhd, a rhodanese-like protein synthesized specifically during sulfur-limited growth (24), was detected within 1 h of S removal (Fig. 6C). The amount of 16S rRNA (Fig. 6D) present in each lane was measured and used to standardize the signals from each sample. The standardized relative levels of the transcripts at each point in the time course are given beneath the autoradiograms.

More extensive quantitative analyses of RNA isolated from cells deprived of N, S, or P are shown in Fig. 7. Washing cells in preparation for dilution into experimental medium caused a reduction in the level of PC mRNA to 55% ($\pm 13\%$) and of APC mRNA to 38% ($\pm 10\%$) relative to that in undisturbed cells (Fig. 7A, open circles and open squares, respectively). The variability in mRNA abundances was due to variability both in the response itself and in the level of each RNA species at the start of the experiment. When cells were placed into complete medium after washing, PC and APC transcripts recovered to prewash levels within 3 h, and the level of mRNA encoding Rhd never increased (Fig. 7A, closed circles). PC and APC mRNA abundances increased slightly after washed cells were placed in S-free medium (Fig. 7B), but fell to their lowest levels of approximately 5 and 10%, respectively, by 2.5 h. mRNA encoding Rhd was detectable within 1 h and reached a maximum after 3 h of S deprivation (Fig. 7B). PC and APC mRNA levels did not increase after washed cells were placed in N-free medium and declined to 5 and 10% of normal levels, respectively, within 1 h (Fig. 7C). No *rhd* expression was detected (not shown). Cells washed and placed in P-free medium (Fig. 7D) regained nearly normal levels of mRNA encoding PC and APC, which were maintained for at least 12 h. Although the timing of the decrease in PC and APC mRNA levels following P deprivation varied among experiments, between 12 and 24 h after P deprivation the abundances of these mRNA species decreased to 15 and 25%, respectively, of normal levels. mRNA encoding an alkaline

FIG. 6. Northern analysis of transcripts encoding PC (A), APC (B), RHD (C), and 16S rRNA (D) from cells that were undisturbed (0), washed (0w), or deprived of S for various times (indicated in hours above the lanes). The same blot was used for all four hybridizations. The percent of each transcript present after normalization to 16S rRNA is shown below each lane. Approximately 3 μ g of total RNA was loaded per lane.

phosphatase (*phoA*), a periplasmic protein synthesized specifically during P deprivation (34), was detected within 4 h of P deprivation and reached maximum levels as the abundances of the PC and APC mRNAs declined (Fig. 7D). The timing of the increase in *phoA* mRNA was also variable and occurred later in cells which also showed a late decrease in PC and APC transcripts. This variability was apparent in the large standard deviations of the mRNA levels at 12 h after P deprivation and probably reflected variable levels of polyphosphate reserves, which can be affected by other aspects of cell physiology (18, 25), within the cells prior to P deprivation.

DISCUSSION

Bleaching of *Synechococcus* spp. in response to nutrient deprivation was first documented in detail by Allen and Smith in 1969 (3). They and other investigators (6, 10, 20, 22, 30, 32, 37, 45) have quantified changes in various cell components during bleaching relative to culture volume, protein, Chl, cell volume, and optical density at 750 nm. The interpretation of such data can be difficult, since these parameters change relative to one another in cells undergoing nutrient stress. For example, optical density at 750 nm only provides an accurate estimation of cell number if the cells being compared are in the same physiological state, since changes in cellular ultrastructure alter the light-scattering properties of cells (3, 9a, 41). We quantified changes on both culture volume and per-cell bases because distinguishing between the total pigment content of a culture and the pigment content of the individual cells that make up the culture aids the interpretation of the data. One shortcoming of this type of analysis is that it does not accommodate changes in cell size. Although Jensen and Rachlin (21)

FIG. 7. Quantitation of mRNA encoding PC (open circles), APC (open squares), RHD (filled circles), and *PhoA* (filled squares) from cells grown in BG-11 (A) or in $-S$ (B), $-N$ (C), or $-P$ (D) media. Note the break in the time axis at 25 h. Values, except those for early controls and for late $-N$ cultures, are the averages of at least two determinations on each of two independent samples. Error bars not shown for *rhd* values in panel B were approximately 30% of the average value, as at 48 h. Other values without error bars had standard deviations that were smaller than the symbols.

reported only small changes in cell size for S- or P-deprived *S. leopoliensis* (*Synechococcus* sp. strain PCC 6301), we have observed that P-deprived cells can be much smaller than nutrient-replete or N- or S-deprived cells (9a).

The conclusion that Chl content remains steady in nutrient-deprived *Synechococcus* spp. (11) is true for the culture as a whole but not for individual cells. Chl content per milliliter and per milligram of protein in N-, S-, and P-deprived cultures remains constant once Chl accumulation ceases (i.e., no net Chl degradation is observed) (Fig. 3A and B) (3, 20). In contrast, cellular Chl content decreases (Fig. 3C and D) (41) because cell division continues after Chl accumulation stops. Both S- and P-deprived cells contained approximately 35% of normal Chl levels upon cessation of growth, despite the fact that Chl synthesis continued for two cell divisions after P deprivation but stopped almost immediately upon S deprivation. N-deprived cells may be unable to undergo more than one division, leaving them with higher Chl levels, although the mechanisms regulating cellular Chl levels are unknown. Bleaching appeared to be less severe in P-deprived cells than in N- or S-deprived cells (the cells were yellow-green rather than yellow), mainly because P-deprived cells showed little evidence of net PBS degradation and maintained a higher PC level, and therefore a higher PC/Chl ratio, than N- or S-deprived cells (Fig. 2 and 3) (20).

Our results show that the PBS degradation process reported in N-deprived cells (46) also occurs during S deprivation. Changes in both rod number and length account for a twofold drop in the PC/APC ratio and for most of the decline in PC content observed within 18 h of N or S deprivation. At later times, the decline in PC content was due to degradation of the remaining PBS structure (cores plus short rods). In contrast, PBS from P-deprived cells changed very little. The decline in 30-kDa linker in PBS from P-deprived cells could be due to the same process occurring in N- and S-deprived cells. However, in P-deprived cells PBS degradation did not take place after PBS accumulation ceased (PC content remained steady for up to 2 weeks of P deprivation [data not shown]). Approximately 75% of the PBS population present after 24 h of P deprivation had been synthesized during growth in P-deficient medium. Hence, the changes in PBS structure during P deprivation may not have required degradation of preexisting PBS but instead may have reflected the synthesis of a new population of smaller PBS.

It has been shown that PBS, which are normally very stable, can be degraded by an unstable proteolytic activity in N-deprived cyanobacteria (11, 43, 44, 46). A similar activity is present in S-deprived cells but may be lacking in P-deprived cells. Because multiple processes are involved in PBS degradation (e.g., loss of the terminal rod hexamer, loss of whole rods, and breakdown of the core and remaining rods), there may be multiple proteases involved in PBS elimination. Both a general and a PC-specific proteolytic activity from *Anabaena* spp. have been described (43, 44), although inactivation of the general protease (27) yields no apparent phenotype.

Previous work (22) showed that PBS synthesis is repressed in N-deprived cells. Bryant (8) reported that PC mRNA was undetectable in N-deprived *Synechococcus* sp. strain PCC 6301. In our studies with *Synechococcus* sp. strain PCC 7942, transcripts encoding PC and APC were present at low levels in N- and S-deprived cells and at slightly higher levels in P-deprived cells. The decrease in mRNA abundance paralleled the cessation of PBS accumulation and occurred after the levels of mRNAs encoding

specific nutrient-acquisition components had risen (Fig. 6 and 7). Reduced transcript accumulation may result from decreased transcription or increased mRNA turnover. Recently, we have determined that the half-life of PC mRNA was reduced from 20.4 ± 2.3 min in nutrient-replete cells to 10.4 ± 3.6 min in S-deprived cells (9a). In contrast, the half-life of APC mRNA remained the same (11.5 ± 2.0 in control cells versus 11.8 ± 5.1 in S-deprived cells). These results suggest that expression of genes encoding PBS components is regulated at the levels of both transcription and transcript stability. PBS biosynthesis may also be regulated at the level of translation. New PC is not accumulated in nutrient-deprived cells after transcript levels fall or in nutrient-replete, stationary-phase (light-limited) cells (2a, 22, 32), although the former maintain 5 to 20% and the latter maintain 80% of normal PBS transcript levels.

PBS degradation would allow the cells to reuse nutrients contained in PBS, which represent up to 50% of cellular protein (e.g., see references 2, 7, 8, and 9). PBS may provide an intracellular store of N (3, 6), especially in marine cyanobacteria, which may often be exposed to N limitation (45; however, see reference 47). However, while the degradation of PBS would provide N to N-deprived cells, PBS contain no Fe or P and would be a poor source of S (37). Boyer et al. (7) proposed that PBS degradation is a consequence of N limitation and that other stresses, particularly Fe limitation, indirectly result in PBS degradation by first causing N limitation. Because PBS contain little S, S deprivation might also be expected to elicit an indirect response. PBS in S-deprived cells are degraded like those in N-deprived cells (Table 1 and Fig. 5), but the mechanism of PBS degradation in Fe-deprived cells has not been examined. Like P-deprived cells, Fe-deprived cells maintain higher PC/Chl ratios (33) than N- or S-deprived cells, so the lower levels of PC in Fe-deprived cells may also be due to PBS dilution by cell division (coupled with the synthesis of smaller PBS) rather than to net PBS degradation.

Since PBS loss appears to be a general aspect of nutrient-limited growth, Reithman et al. (35) proposed that a mechanism common to different nutrient-limited conditions triggers PBS degradation. However, we have shown that PBS degradation and loss are not necessarily the same process, since extensive proteolysis of PBS did not occur in P-deprived cells (as it did in N- or S-deprived cells), although cellular PBS content declined. It is not clear whether N and S deprivation generate the same general signal for initiating the destruction of PBS and repression of new PBS synthesis or whether these processes are triggered by independent, nutrient-specific pathways, such as those which must control the expression of nutrient acquisition genes (e.g., *phoA* and *rhd*). If nutrient-specific pathways are responsible for controlling PBS size and stability, those starting from N or S deprivation appear to join, while that from P deprivation appears to be independent. An example of nutrient-specific alteration of PBS biosynthesis does occur in *Fremyella diplosiphon*, which has a specific PC gene set that is expressed only during S-limited growth (28). Alternatively, an intracellular signal which triggers PBS degradation may be generated in N- and S-deprived cells but not in P-deprived cells. Recently, we have isolated mutants that do not degrade the PBS during N or S deprivation. The gene which contains the lesion in mutant strains is expressed at high levels during both N and S deprivation, but its mRNA is barely detectable in nutrient-replete or P-deprived cells (9a). These results support the conclusions of the physiological and biochemical studies presented here, which suggest that

the bleaching response of N- and S-deprived cells is different from that of P-deprived cells.

Both PBS degradation and synthesis may be regulated differently under P deprivation than they are under N or S deprivation, because P is most likely to be the limiting nutrient in the freshwater ecosystems from which the ancestor of *Synechococcus* sp. strain PCC 7942 was isolated (42). Therefore, the cells may possess special strategies to survive P-limiting conditions. One such strategy, the storage of polyphosphate reserves, may allow cells to withstand short periods of P deprivation without major changes in cell growth or physiology, as during the first 12 h of P deprivation in this study. Because limitation for N or S is less likely to occur in this organism's natural environment, the cells may be less prepared to cope with such conditions (*Synechococcus* spp. do not appear to possess cyanophycin, a N storage compound common in cyanobacteria [2, 39]), and a more severe response may be elicited. It is not clear whether cells deprived of an essential nutrient enter a "dormant" state, although we have observed that a small percentage of nutrient-deprived *Synechococcus* cells can survive in the chlorotic state for weeks (9a).

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