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Protein Synthesis and Proteolysis in Immobilized Cells of the Cyanobacterium *Nostoc commune* UTEX 584 Exposed to Matric Water Stress

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Cells of the cyanobacterium *Nostoc commune* UTEX 584 in exponential growth were subjected to acute water stress by immobilizing them on solid supports and drying them at a matric water potential (ψ_m) of −99.5 MPa. Cells which had been grown in the presence of $Na_2{}^{35}SO_4$ before immobilization and rapid drying continued to incorporate ^{35}S into protein for 90 min. This incorporation was inhibited by chloramphenicol. No unique proteins appeared to be synthesized during this time. Upon further drying, the level of incorporation of ^{35}S in protein began to decrease. In contrast, there was an apparent increase in the level of certain phycobiliprotein subunits in solubilized protein extracts of these cells. Extensive proteolysis was detected after prolonged desiccation (17 days) of the cells in the light, although they still remained intact. Phycobilisomes became dissociated in both light- and dark-stored desiccated material.

Recognition of the marked and often complex changes in enzyme biosynthesis that take place within cells of *Escherichia coli* when they are subjected to a shift in growth conditions has prompted interest in stimulons, that is, sets of genes that become active and produce their protein products in response to particular environmental stimuli (2, 10, 20). For example, a change in the osmotic water potential of the growth medium can influence the differential synthesis of two outer membrane proteins OmpC and OmpF (12, 18). A model has also been proposed whereby a 95K protein, the *kdpD* product, interacts with the promoter of the *kdp* operon in response to a rise in turgor pressure. Induction of the *hop* regulon, in response to a decrease in the osmotic water potential of the growth medium, leads to an elevated rate of synthesis of more than three major proteins. In this last case, the response is considered to be a true adaptation to a change in environmental conditions rather than a general response to cell damage (8). It is expected that studies such as these will reveal novel patterns, if not novel mechanisms, of gene regulation (14).

Clearly, our current knowledge and understanding of gene expression in procaryotes derives almost exclusively from investigations of the growth of heterotrophic eubacteria, particularly *Escherichia coli*. In contrast, the molecular biology of cyanobacteria is poorly understood despite the utility of these procaryotes as model systems for the study of nitrogen fixation, differentiation, and photosynthesis (11, 13).

Many cyanobacteria express a marked tolerance of water stress and desiccation (6, 25, 27, 28, 32). In preliminary studies with immobilized cells of the filamentous, nitrogen-fixing strain *Nostoc commune* UTEX 584, we described upshifts and downshifts in both nitrogenase activity and the size of the intracellular ATP pool in response to matric water stress (25, 26; M. Potts, N. S. Morrison, *Proceedings of the 3rd International Symposium on N_2 Fixation With Non-Legumes*, Helsinki, in press).

This study focuses on protein turnover in immobilized cells of *N. commune* UTEX 584 when they are subjected to acute water stress (rapid drying at a matric water potential [ψ_m] of −99.5 MPa).

MATERIALS AND METHODS

Growth of cells. *N. commune* UTEX 584 was grown in BG-11_o (31) medium in continuous axenic culture with an air-lift fermentation system (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The volume of the reactor vessel was 1.8 liter. Cells were grown under a continuous photon flux density of 300 μmol of photons m^{-2} s^{-1} at 32°C and at a pH of 8.7.

Labeling of cells and measurement of protein synthesis. Cells were harvested directly from the fermentor, washed extensively in BG-11_{o-s} (BG-11_o that lacked combined sulfur), homogenized briefly to disperse any filament aggregates, and suspended in the same medium to a cell density of approximately 60 μg of total protein ml^{-1}. The cell suspension (4 ml) was then aerated vigorously under a photon flux density of 300 μmol of photons m^{-2} s^{-1} at 32°C. After equilibration for 15 to 30 min, 160 μCi of $Na_2{}^{35}SO_4$ (80 μl, carrier free; specific activity, 1,498 Ci $mmol^{-1}$) was added to the suspension, and the incubation was continued. Samples of 50 μl were removed periodically during the incubation and transferred to Whatman no. 3MM filter disks (23-mm diameter). The incorporation of ^{35}S into protein was measured by the method of Mans and Novelli (19).

The incorporation of ^{35}S into protein during subjection of the cells to matric water stress was determined after the addition of label to a cell suspension either 60 min or immediately before immobilization and rapid drying of the cells at −99.5 MPa (see below).

Immobilization of cells and control of matric water potential. After a period of labeling, samples (50 or 100 μl) of a cell suspension were transferred to Whatman 3MM filter disks (23-mm diameter) which were supported on steel pins. Depending on the experiment, the filters, at the time of cell immobilization, were either dry, saturated with BG-11_o, or saturated with BG-11_{o-s}. Filters were incubated at 32°C under a photon flux density of 300 μmol photons m^{-2} s^{-1}, at a matric water potential of −99.5 MPa (26). The filters were

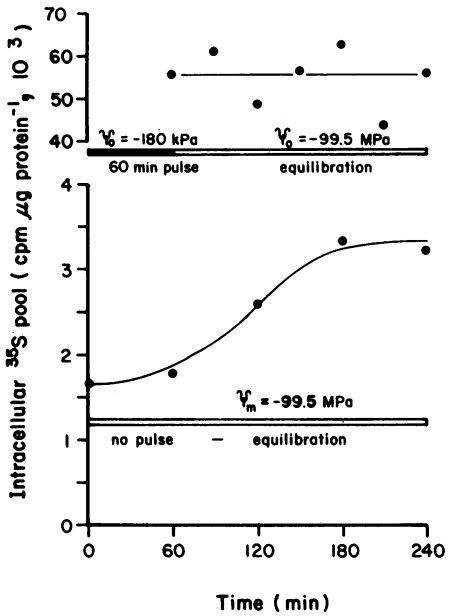

FIG. 1. Change in the size of the total intracellular pool of ^{35}S after immobilization of cells. $^{35}SO_4$ was added to a suspension of cells (at a density of 69.9 μg of total protein ml^{-1}) in exponential growth. One series of samples (100 μl) was immobilized immediately after the addition of ^{35}S, and a second series was immobilized after the cell suspension had been incubated for 60 min in the presence of ^{35}S. In each case the filters were dry before the immobilization of the cells, and incubation was continued at −99.5 MPa. Periodically, filters were removed from the incubation chamber and then washed extensively in distilled water at 4°C. After drying, the filters were placed directly in scintillation fluid to measure radioactivity. All values were adjusted for quenching and nonspecific uptake of ^{35}S during washing.

allowed to equilibrate to this matric water potential, or they were moistened periodically with 50 μl of BG-11$_o$ or BG-11$_{o-s}$.

After a period of drying and equilibration, the filters were processed to measure the incorporation of ^{35}S in protein (see above). When larger quantities of cell material were required, the total cell suspension was centrifuged at 12,000 × *g* for 5 min, and the pellet was spread as a thin veneer on a fine-mesh nylon filter support (26). The advantage of this support over filter disk supports was the efficiency of recovery of the desiccated cell material.

Measurement of the total uptake of ^{35}S by cells. The intracellular pool of ^{35}S in cells was measured after extensive washing of filters in ice-cold H_2O (in certain experiments, BG-11$_o$ was used).

Radioactivity that remained on filters after the various manipulations was measured in Biofluor cocktail (New England Nuclear Corp., Boston Mass.) with a Packard Tri-Carb model 2405 liquid scintillation counter. Measurements were corrected for quenching and nonspecific adsorption of ^{35}S to filters.

Measurement of total protein. Total protein was extracted and measured by the methods of Kochert (15, 16).

Extraction of soluble proteins. Cells were ground with sterile sand in a chilled mortar at 0 to 4°C, and the soluble proteins were extracted in cold buffer [10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid [pH 7.2], 10 mM NaCl, 5mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaN_3). The extracts were cleared by centrifugation at 12,000 × *g* for 10 min, mixed with an equal volume of trichloroacetic acid (20%, wt/vol), and kept on ice for at least 45 min. The precipitates were collected by centrifugation and then suspended in trichloroacetic acid (5%, wt/vol). After washing in ice-cold acetone-water (50:50, vol/vol) and then ether, the pellets were dried under a stream of air and solubilized in cracking buffer (0.2 M Tris, [pH 8.8], 2% [wt/vol] sodium dodecyl sulfate, 1 M β-mercaptoethanol, 15% [vol/vol] glycerol, 0.01% [wt/vol] bromphenol blue) at 100°C for 3 min.

Electrophoresis of protein extracts. Samples were analyzed on either 10 or 15% (wt/vol) polyacrylamide gels supported on gel bond (FMC Corp., Rockland, Maine) with a vertical-gel apparatus (Bethesda Research Laboratories) and the buffer system of Laemmli (17). After fixing, gels were stained with either Coomassie brilliant blue R or silver stain, or they were prepared for fluorography by impregnation with En^{3}Hance solution, (New England Nuclear). In the last case, proteins were detected with Kodak X-Omat AR (XAR-5) film exposed at −70°C.

Extraction of phycobilisomes. Cells were ground with sand in cold grinding buffer (0.65 M NaH_2PO_4-K_2HPO_4 [pH 8.0], 1 mM β-mercaptoethanol). The extracts were incubated with 1.2% (vol/vol) Triton X-100 for 30 min at room temperature and were then centrifuged at 31,000 × *g* for 30 min. Purified phycobilisomes and additional fractions were obtained after centrifugation of the aqueous extracts through 0.2 to 1.0 M sucrose step gradients, at 23,000 × *g*, for 13 h in a Beckman SW27 rotor (37). The absorbance spectrum of phycobilisome preparations and other fractions isolated from gradients was measured with a Hewlett Packard model 8450A UV-visible spectrophotometer with ancilliary 7470 graphics plotter. The protein composition of these samples was analyzed after solubilization in cracking buffer (room temperature) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (see above).

RESULTS

Under the experimental conditions employed during incorporation experiments, the time required for immobilized cells to reach equilibrium, that is, the time after which no change in weight of the cells could be detected, depended on the volume and density of the cell suspension that was transferred to a filter and the physical nature of the solid support. Upon transfer of a 50-μl sample of well-dispersed cell suspension to a dry Whatman 3MM filter (23-mm diameter), the filaments were immobilized immediately within the confines of the upper matrix of the support and occupied a circular area approximately 8 mm in diameter. The liquid medium in association with the cells was absorbed rapidly and wetted the filter in a circular area approximately 1 cm in diameter. When the filter was then incubated at −99.5 MPa and illuminated (photon flux density of 300 μmol of photons m^{-2} s^{-1}) at 32°C, it was dry to the touch within less then 3 min. When the filter was saturated with liquid medium before the immobilization of the same quantity of cell suspension, the support appeared dry within 30 to 40 min. Generally, filters that supported different quantities of cell suspension (between 0.16 and 2.7 g [wet weight]) achieved a constant weight after 80 to 90 min of incubation at −99.5 MPa (data not shown).

Uptake of $^{35}SO_4$ by immobilized cells. The total intracellular pool of ^{35}S in cells that were immobilized immediately after the addition of $^{35}SO_4$ to a cell suspension increased during a drying period (ψ_m = −99.5 MPa) of 4 h (Fig. 1). The trend involved a slight increase in the size of the pool during

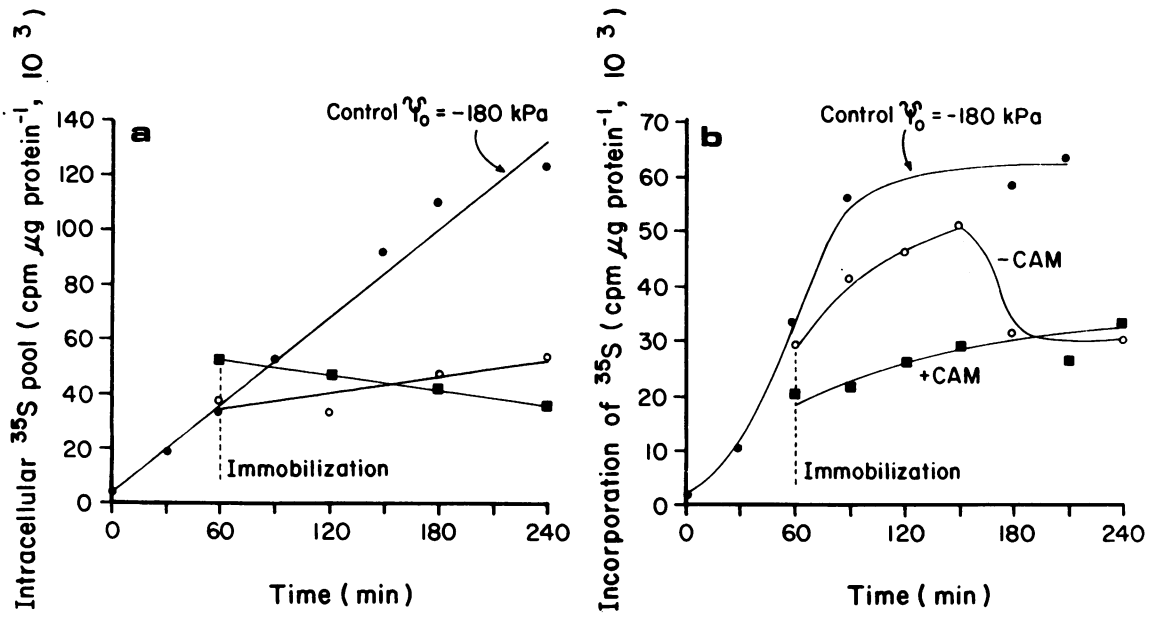

FIG. 2. (a) Change in the size of the total intracellular pool of ^{35}S after immobilization of cells at −99.5 MPa. (●) Increase in the total intracellular pool of ^{35}S in cells growing exponentially in liquid culture. (○) Size of the intracellular ^{35}S pool after 60 min of incubation in the presence of $^{35}SO_4$ and immobilization on dry filters at −99.5 MPa for different periods of time. Filters were washed extensively in BG-11$_o$ (supplemented with 0.1 M $MgSO_4$). (■) Cells were immobilized on filters that were saturated with BG-11$_o$ before immobilization of the cells. Filters were then washed extensively in BG-11$_o$ (supplemented with 0.1 M $MgSO_4$) before measurements of radioactivity were taken. (b) Level of incorporation of ^{35}S in protein after immobilization of cells at −99.5 MPa. (●) Incorporation of ^{35}S by a suspension of cells in exponential growth. (○) Incorporation by cells after 60 min of incubation in the presence of ^{35}S and then immobilization on filters that were presoaked in BG-11$_{o-s}$. Incubation was at −99.5 MPa. (■) Cells were immobilized in the presence of 50 µg of chloramphenicol ml^{-1}.

the first 60 min of immobilization and then a linear increase during the following 2 h. After this time the pool size remained constant. After 3 h, the size of the pool was less than 10% of that measured in cells grown in liquid culture (ψ_o = −180 kPa) for 60 min before immobilization. In this case, the size of the pool was maintained after immobilization of the cells and during a 3-h period of drying at −99.5 MPa, although there was greater variability between measurements. The data illustrated in Fig. 1 were obtained when the size of the total intracellular pool of ^{35}S was measured after the cells had been washed extensively in distilled water at 4°C (see Materials and Methods). When BG-11$_o$ (supplemented with 0.1 M $MgSO_4$) was used in the washing process, the variability in readings, particularly in cases where the pool size was large, was far less (Fig. 2a). In addition, an increase in the pool size was observed during drying when prelabeled cells were immobilized on dry filters, whereas the pool size decreased in the same material when the cells were immobilized on filters that were saturated previously with BG-11$_o$. In contrast to the pattern of $^{35}SO_4$ uptake shown by immobilized cells (Fig. 1) after the addition of $^{35}SO_4$ to a suspension of cells in exponential growth, there was a linear increase in the size of the intracellular pool of ^{35}S with no lag during a 4-h incubation (Fig. 2a).

Protein synthesis by prelabeled immobilized cells. When cells were grown in the presence of $^{35}SO_4$ for 60 min and then immobilized on dry filters and dried rapidly, the cells continued to incorporate ^{35}S into protein for approximately 90 min, after which time a net decrease in the level of incorporation was observed (Fig. 2b). This incorporation of ^{35}S into protein was inhibited by chloramphenicol. The maximum level of incorporation was detected in immobilized cells at a time when, as judged from dry weight measurements, they had lost the bulk of their associated water. In one experiment, the level of incorporation at time 150 min, that is, after 90 min of drying, was higher than that measured in cells grown in liquid culture for an equivalent period. The level of incorporation in the dried cells fell to preimmobilization levels after 130 min at −99.5 MPa. A high level of incorporation was still detectable after 24 h of drying.

Protein synthesis of unlabeled immobilized cells. When cells were immobilized immediately after the addition of $^{35}SO_4$ to the suspension and then subjected to a matric water potential (ψ_m) of −99.5 MPa, no significant incorporation of ^{35}S into protein was detected during a 4-h incubation period. A low level of incorporation was detected during the 60 min after immobilization of cells when the filter supports were kept moist with periodic additions of BG-11$_{o-s}$ ($\psi_m + \psi_o$ = −180 kPa). After 60 min of incubation the level of incorporation then decreased. This initial increase in the level of incorporation represented only 7% of that detected during the same time period when the cells were incubated under osmotic conditions with aeration (i.e., in suspension; ψ_o = −180 kPa).

Proteolysis during equilibration of immobilized cells. In view of the results of incorporation experiments (see above), a qualitative electrophoretic analysis was made of the proteins present in immobilized cells during short- and long-term drying at −99.5 MPa.

Figure 3 illustrates the patterns which were obtained after electrophoresis of solubilized proteins extracted from control cells and material dried at −99.5 MPa for either 2 or 24 h. No obvious differences could be detected among the three samples. The most prominent proteins were the phycobiliprotein subunits located in the lowest region of the gel and a 50K protein. When identical gels were treated with silver stain, four bands, corresponding to 141, 158, 186, and 206K proteins, were obvious in the protein extract from cell

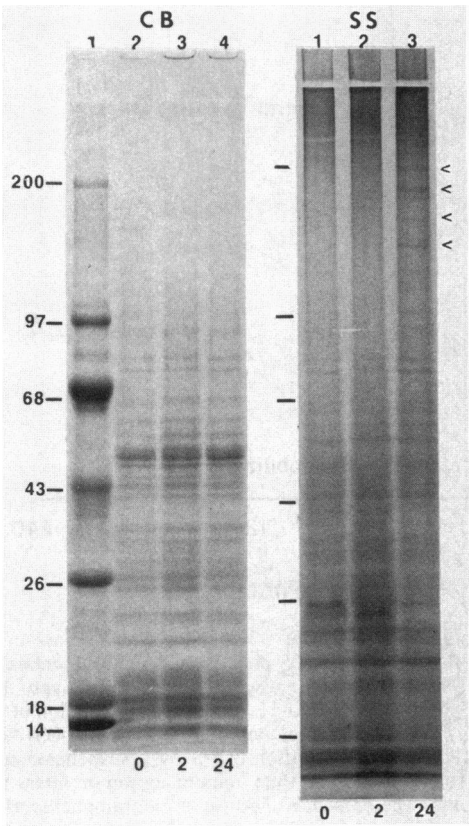

FIG. 3. Polyacrylamide gel electrophoretic analysis of solubilized protein extracts obtained from cells in liquid culture and the same material immobilized and dried at −99.5 MPa for either 2 or 24 h. Gels were stained with either Coomassie brilliant blue R (CB) or silver stain (SS). CB lanes: 1, molecular weight standards; 2, control; 3, cells dried for 2 h; 4, cells dried for 24 h. SS lanes: 1, control; 2, cells dried for 2 h; 3, cells dried for 24 h.

material desiccated for 24 h in the light. These bands were not apparent in protein extracts from control material or material desiccated for 2 h in the light. Streaking and smearing of bands were observed upon electrophoresis and fluorographic analysis of extracts obtained from cells that had been labeled with ^{35}S and then immobilized and dried at −99.5 MPa for 17 h (Fig. 4). Further, in comparison to the patterns obtained after electrophoretic analysis of control material, the following changes were observed: a decrease in intensity of a band at 59K, a decrease in mobility of a protein of 20K, the appearance of a new band at 46K, and an increase in intensity of a band that corresponds to a 17.4K biliprotein. Smearing and streaking in gel lanes were pronounced after the analysis of protein extracts taken from material that had been desiccated for 15 days (data not shown). In this material only some dozen proteins were apparent after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The majority of these proteins appeared to have decreased mobilities as judged from a comparison of the banding pattern with that obtained for control samples. The most obvious protein was a 60K protein.

Changes in phycobilisomes during drying of immobilized cells. As noted previously (25), visible differences in color were apparent in cells that had been stored under different conditions during desiccation. The colored phycobiliproteins represent a major fraction of the protein present in cyanobacterial cells. An analysis was made, therefore, of the effects of drying on the phycobilisome content of immobilized cells.

After cell disruption and treatment of the extracts with Triton X-100, supernatants from cells that were desiccated for 18 days, either in the light or the dark, appeared purple to grey-blue, in contrast to the supernatant of the control, which was brown-red. After centrifugation at 31,000 × *g* for 30 min, the supernatants from the control and dark-stored cells were both blue-purple in color, whereas the supernatant from the light-stored cells was bright pink.

The absorbtion spectrum of these samples revealed three major peaks at 564, 614, and 660 nm. These correspond to the absorption maxima of three phycobiliproteins: phycoerythrin, phycocyanin, and allophycocyanin, respectively. The content of phycobiliprotein in desiccated cells was lower than that found in control cells. In addition, the phycobiliprotein content of light-stored cells was lower than that of dark-stored cells.

The results obtained when these supernatants were subjected to sucrose density centrifugation are illustrated in Fig. 5a. A shallow green layer of solubilized membrane material was observed at the surface of the control gradient. This layer was less obvious at the surface of gradient D (cell material desiccated in the dark) and was not apparent in gradient L (cell material desiccated in the light). A series of four bright blue bands, corresponding to intact phycobilisomes, was observed in the 0.6 to 0.8 M sucrose region of the control gradient. Three bands were observed in gradient D; in relation to the control, the banding pattern showed a decrease in polydispersity and sedimentation rate. These blue bands were absent from gradient L. No obvious qualitative differences were observed between the electro-

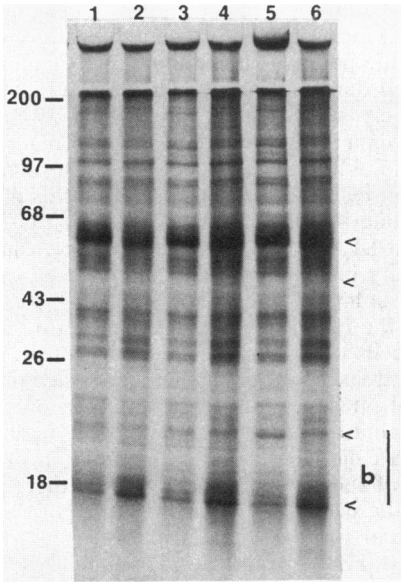

FIG. 4. Fluorographic analysis of solubilized protein extracts obtained from cells grown in suspension in the presence of ^{35}S for 4 h (26,816 cpm per μg of total protein) and from the same material after immobilization, rapid drying, and incubation at −99.5 MPa for 17 h. The biliprotein subunits are indicated (b). The amount of radioactivity loaded in the well of each lane is indicated. Control lanes: 1, 3, and 5; 51,351, 51,351, and 102, 702 cpm, respectively. Cells dried for 17 h: lanes 2, 4 and 6; 56,421, 112,842, and 112,842 cpm, respectively.

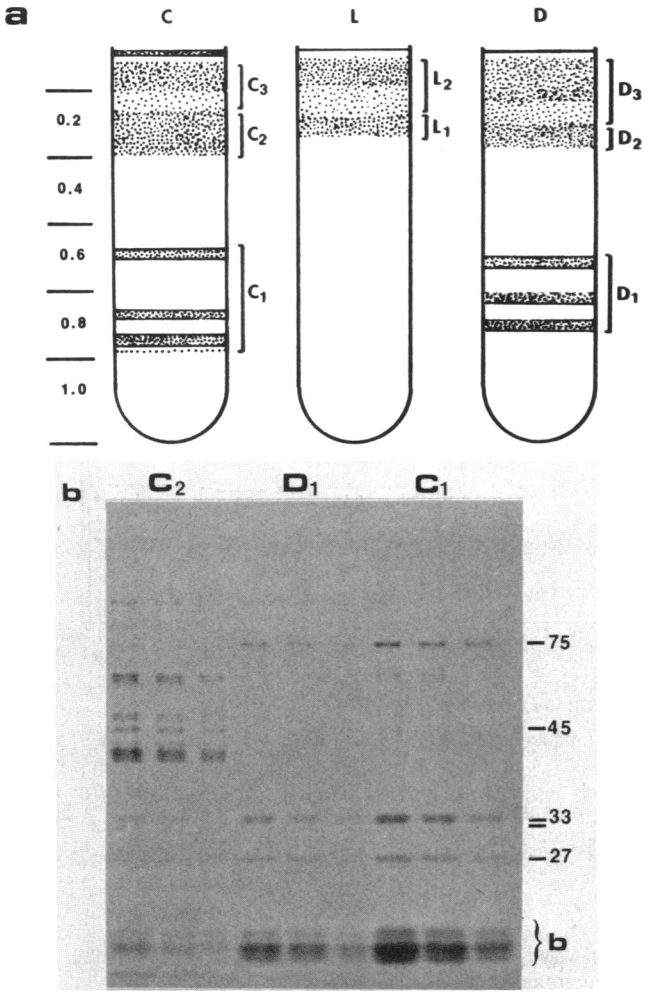

FIG. 5. Influence of desiccation on phycobilisomes. (a) Sucrose density gradient analysis of extracts: C, control material; L, cell material desiccated in the light; D, cell material desiccated in the dark. (b) Polyacrylamide gel electrophoretic analysis of fractions C_1, C_2, and D_1. Samples were analyzed on a 10% (wt/vol) acrylamide gel, and gels were stained with Coomassie brilliant blue R. The molecular masses (in kilodaltons) of the major phycobilisome linker polypeptides are indicated. The biliprotein subunits (b) are located in the lower portion of the gel photograph.

phoretic profiles of solubilized phycobilisomes isolated from gradient D (D_1) or the control (C_1; Fig. 5b).

A diffuse zone of free phycobiliproteins and some membrane material occupied the upper region of all three gradients. In each gradient this zone appeared to have two components: an upper portion which was pink (fractions C_3, L_2, D_3) and a lower portion which was purple-pink (fractions C_2, L_1, D_2). The lower limit of the zone corresponded approximately with the lower part of the 0.2 M sucrose layer (Fig. 5a). Fractions obtained from the lower portion of the zones in gradients L and D contained relatively large amounts of free phycobiliproteins (Fig. 6). The same fraction from the control gradient contained a very small proportion of phycoerythrin and phycocyanin, but approximately the same amount of allophycocyanin as seen in the equivalent fractions from the other two gradients (lane C_2, Fig. 5b). The upper region of the control gradient was enriched in allophycocyanin, whereas free phycoerythrin and phycocyanin dominated the upper region of gradients L and D.

DISCUSSION

When cells alter the synthesis of their proteins in response to a particular stimulus, it is essential to distinguish between changes that may be considered a consequence of cell damage or senescence and those that may represent a true adaptation of the cell (1, 36). In this respect, the influence of water stress on gene expression is poorly understood. Osmotic upshifts in *E. coli* have been shown to induce the *hop* regulon as well as cause changes in the abundance of membrane proteins; the hop products are distinct from the heat-shock proteins. The effects of osmotic water stress on cyanobacteria are currently the focus of extensive investigation (5, 22, 29, 30). Studies on the effects of matric water stress on these microorganisms are few (6, 25, 27). Desiccation tolerance and matric water relations of cells have been studied almost exclusively in eucaryotic systems and especially thoroughly by Bewley and co-workers (1, 23, 24, 34).

Whereas our previous work demonstrated the marked capacity of *N. commune* to tolerate desiccation and acute water stress, the observation of a relatively high rate of incorporation of ^{35}S into protein in cells that had been interrupted during logarithmic growth in liquid culture, immobilized, and then dried rapidly at −99.5 MPa was surprising. Superficially, this response would seem to typify the sluggish shift that these microorganisms often undergo in response to pronounced fluctuations in growth conditions (7, 11). One-dimensional electrophoresis could not detect any marked accumulation of specific proteins after 2 h of drying. In addition, two-dimensional fluorographic analysis indicates there are few qualitative changes in the pool of ^{35}S-labeled protein after rapid drying and incubation at −99.5 MPa for 30 min (unpublished data). These findings are similar to those observed for desiccation-tolerant mosses (24). Moss proteins are remarkably stable in vivo during desiccation and rehydration, with no selective loss as a consequence of the stress. However, a previous study demonstrated that nitrogenase activity in *N. commune* ceased within 20 min of immobilization (25, 26); heterocysts seem more sensitive to water stress than vegetative cells (3, 25). It is not known whether nitrogenase is destroyed or inactivated during rapid drying, or whether another component of the nitrogen-fixing complex is affected. In the case of a rapid-dried moss, drying may take 15 to 60 min, and this is thought to be insufficient time to permit the synthesis of new proteins (24). Nevertheless, it has been suggested that an accumulation of hydrophobic proteins (rich in -SH groups) may maintain a reduced environment within cells; this, in turn, may prevent protein aggregation or denaturation (35). The choice of isotope (^{35}S, ^{14}C, or ^{3}H) may therefore be an important consideration in the design of experiments to assess the occurrence of proteins that show either sensitivity or tolerance to matric water stress.

As shown in this study, sulfate uptake, an energy-dependent process, can occur after immobilization and during drying of cells, although at a markedly lower rate than measured in cell suspensions. In experiments where incorporation of ^{35}S into protein was shown to persist for some time after immobilization and rapid drying of the cells, it is clear that the ^{35}S was derived from the intracellular pool. As such, it is possible that the finite pool size of ^{35}S was one factor limiting the rate of incorporation of ^{35}S into protein during drying of the cells.

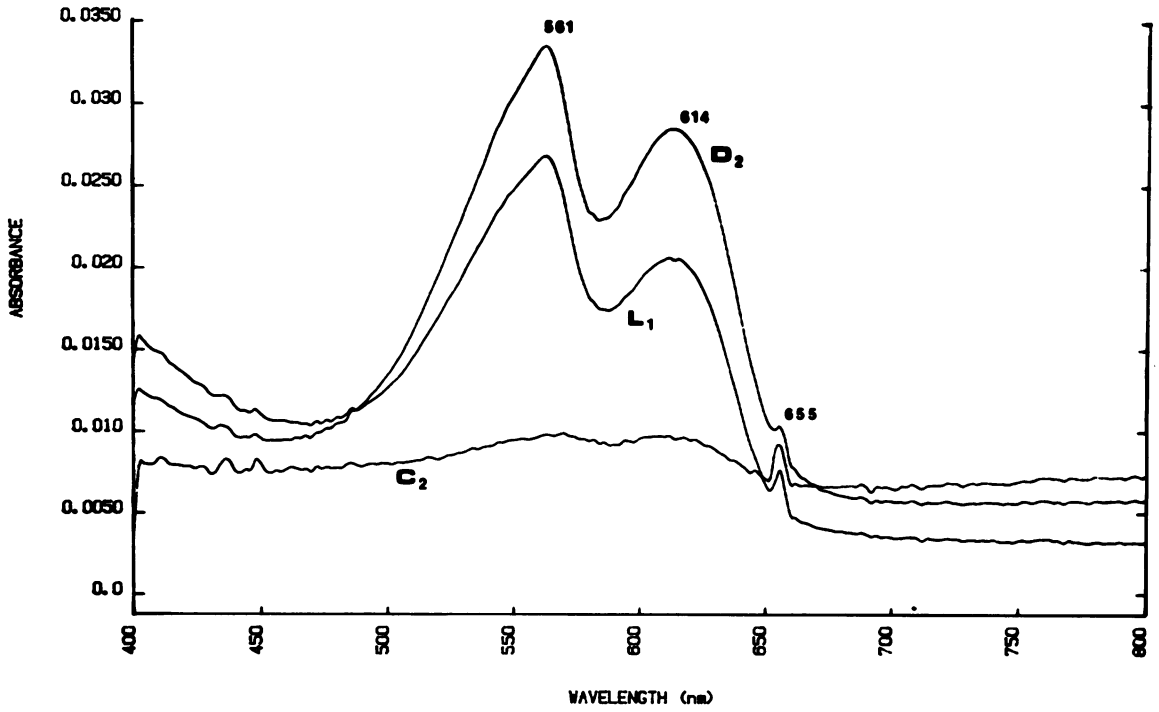

FIG. 6. Absorption spectra of fractions C_2, L_1, and D_2 obtained from sucrose density gradients (see legend to Fig. 5a).

After 90 min of drying the first indication of proteolysis—at least of ^{35}S-labeled protein—was observed. After this time it is difficult to assess the relative importance of proteolysis due to desiccation or proteolytic effects due to photooxidation. Clearly, extended desiccation leads to pronounced protein degradation in response to light-dependent effects, but, as pointed out above, it is hard to assess the potential significance of such findings when one considers the response of natural populations of cyanobacteria to desiccation (25, 32). In both light- and dark-stored cells there is an increase in free phycobiliproteins. After desiccation of cells for only a relatively short period, there is an obvious increase in the level of a 17K protein, possibly α-allophycocyanin (Fig. 4). Although intact phycobilisomes appeared to be sensitive to desiccation, relatively large quantities of phycobiliproteins were still detected in dark-desiccated cells. Previous studies have indicated that such cells also remain intact after extensive and prolonged photoxidation, and their activity (nitrogenase; ATP generation) is restored by rewetting and after a lag that involves protein synthesis (25, 33; Potts et al., in press).

The present study demonstrates that protein synthesis is maintained at a high level in *N. commune* during the time cells are subjected to acute water stress, but no unique class of proteins appears to be synthesized. In this latter respect the response of *N. commune* to matric water stress (a shock) is different from the responses shown to heat shock and osmotic shock by other microorganisms (4, 8, 9, 21). It should be noted, however, that this study was concerned with the effects of rapid drying on cells in logarithmic growth. In other systems, slow drying has been shown to have more damaging effects on cells (1, 23, 24).

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