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Changes in Sulfate Transport Characteristics and Protein Composition of *Anacystis nidulans* R2 during Sulfur Deprivation†

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Sulfur-starved cells of *Anacystis nidulans* have an increased capacity to take up sulfate. The apparent V_{max} for sulfate uptake increased at least 10-fold after 24 h of sulfur deprivation, whereas the $K_{1/2}$ remained unchanged at approximately 1.35 μM. The initial rate of sulfate uptake increased between 2 and 6 h after transfer of the cells to sulfur-free medium, in concert with elevated levels of three cytoplasmic membrane polypeptides with molecular masses of 43, 42, and 36 kilodaltons (kDa). The amounts of these polypeptides did not increase in response to nitrogen or phosphorus deprivation. A fourth cytoplasmic membrane polypeptide of 17 kDa did not appear until 24 h after transfer to sulfur-deficient medium. In the total soluble fraction, three polypeptides with masses of 36.5, 33.5, and 28.5 kDa increased dramatically in response to sulfur deprivation, but not in response to nitrogen or phosphorus deprivation. The specificity and abundance of these polypeptides indicate that they could play an important role in the response of *A. nidulans* to sulfur deprivation.

Nutrient levels can be quite variable in freshwater environments and can influence the growth and distribution of aquatic organisms (5). Although phosphorus is generally the limiting nutrient in freshwater systems (14), *Anacystis nidulans*, a freshwater, unicellular cyanobacterium, exhibits dramatic changes in both its morphological and its physiological characteristics in response to sulfur deprivation. When cells of *A. nidulans* are deprived of a sulfur source, internal stores of sulfur are depleted, growth stops, and the cells become bleached; the accessory photosynthetic pigments phycocyanin and allophycocyanin are almost completely degraded, and chlorophyll is degraded to a variable extent (15, 18). Similar bleaching occurs in response to starvation for nitrogen (1) and phosphorus (6). Ultrastructural changes which accompany sulfur deprivation include cell wall thickening (8) and a marked reduction of the thylakoid membranes. Furthermore, large quantities of glycogen, polyphosphate, and poly-β-hydroxybutyric acid accumulate in electron-dense inclusions in the cytoplasm of the cell (11, 18). Although sulfur-deprived cells stop growing, cultures can survive at least 2 days of starvation with little loss of cell viability (15; unpublished data).

The physiological changes which accompany sulfur deprivation may enable *A. nidulans* to compete more effectively for low levels of exogenous sulfur. Within 7 h of deprivation, sulfate transport capacity in *A. nidulans* is elevated 10-fold (7). This transport is light dependent, inhibited by metabolic poisons (*N*, *N'*-dicyclohexylcarbodiimide and carbonyl cyanide *m*-chlorophenylhydrazone) and sulfate analogs (sulfite, thiosulfate, chromate, and selenate), and occurs against an electrical gradient as indicated by trimethylphenylmethylphosphonium partitioning (7, 17). Elevated rates of sulfate transport which accompany sulfur deprivation can be blocked by chloramphenicol, suggesting that protein synthesis is required for acclimation to occur (7).

Whereas the physiological and ultrastructural changes accompanying sulfur deprivation in *A. nidulans* have been documented, little is known about what changes in protein synthesis and gene expression occur over the course of acclimation. In *Saccharomyces cerevisiae* (2) there are multiple sulfate transport systems; one is constitutively expressed and has a low affinity for sulfate, and a second, high-affinity system is synthesized when exogenous sulfur levels fall. *Salmonella typhimurium*, on the other hand, has a single sulfate transport system which increases in abundance when the cells are grown with limiting sulfur (3). To determine whether there is a high-affinity transport system which is synthesized in response to sulfur deprivation in *A. nidulans*, we have compared the uptake kinetics of starved and unstarved cells. In parallel with kinetic measurements, we have examined the soluble cellular proteins and the polypeptide composition of purified cytoplasmic membranes during the course of sulfur deprivation and have found that several different polypeptides increase in abundance.

MATERIALS AND METHODS

Culture conditions. *A. nidulans* R2 (*Synechococcus* sp. strain 6301) was obtained from the laboratory of Louis Sherman. Cells were grown in both liquid and solid (1.5% agar) BG-11 (16) medium buffered to pH 8 with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEP ES). Liquid cultures were grown in 50-ml glass tubes, bubbled with air enriched to 3% CO_2, and maintained at 30°C. Illumination was from incandescent bulbs at 100 μmol/m^2 per s.

For nutrient deprivation experiments, cells were pelleted from the medium at 3,500 × *g* for 5 min at room temperature, washed once with sulfur-deficient medium, and resuspended in sulfur-deficient medium to a final concentration of 1×10^7 to 5×10^7 cells per ml. For sulfur-deficient medium, $MgSO_4$ was replaced with $MgCl_2$ (final concentration, 300 μM), and the trace elements used contained $ZnCl_2$ and $CuCl_2$ in place of $ZnSO_4$ and $CuSO_4$, respectively. For phosphorus-deficient medium, K_2HPO_4 was replaced with KCl to an equivalent potassium concentration. For nitrogen-deficient medium, NaCl and $FeCl_3$ replaced $NaNO_3$ and ferric ammo-

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nium citrate, respectively. Control trace elements were used to make the final nitrate concentration 0.34 μM.

Sulfate uptake assays. Cells were harvested as for transfers, washed once with sulfate-deficient BG-11 medium, and resuspended in the same medium to a concentration of 1×10^7 to 5×10^7 cells per ml. Portions (1 or 2 ml) of this suspension were placed in a water-jacketed glass chamber maintained at 30°C and illuminated with a slide projector at 150 μmol/m^2 per s. The cells were preequilibrated under these new conditions for 2 min before sulfate uptake was measured. Uptake was started by the addition of $MgSO_4$ (final concentration, 0.1 to 10 μM) containing 5 to 45 μCi of carrier-free $H_2{}^{35}SO_4$ (New England Nuclear Corp.). Samples (100 μl) were withdrawn, and the cells were collected on 0.45-μm-pore-size GA-6 cellulose acetate filters (Gelman Sciences, Inc.) that had been prewetted with wash solution (20 μM $MgSO_4$, 5 mM HEPES, pH 8.0) and washed twice with 1 ml of wash solution. Filters were transferred to glass vials and suspended in 5 ml of Aqueous Counting Solution (Amersham Corp.) for scintillation counting. The number of cells per sample (determined with a hemacytometer) and the specific activity (counts per minute per femtomole) of the $^{35}SO_4{}^{2-}$ stock solution added were used to calculate total sulfate uptake (femtomoles per 10^6 cells). The slope of the regression line through the first three or four time points (15, 30, 45, and 60 or 90 seconds after addition of $^{35}SO_4{}^{2-}$) yielded the initial rate of uptake for each experiment.

Cell fractionation. For each cytoplasmic membrane preparation, cells from 300 ml of culture at approximately 10^8 cells per ml were used. A cell homogenate was prepared as described by Omata and Ogawa (13) and was made 50% sucrose by the addition of 0.74 volume of 90% sucrose–10 mM TES [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] sodium hydroxide (pH 7.0)–10 mM NaCl–5 mM EDTA. Portions (6 ml) were transferred to each of three 14-ml ultracentrifuge tubes. Over this was layered 2.7 ml of 39% sucrose, 1 ml of 30% sucrose, and 2.3 ml of 10% sucrose. All sucrose solutions also contained 10 mM TES-sodium hydroxide (pH 7.0)–10 mM NaCl–5 mM EDTA. The tubes were centrifuged at 130,000 × *g* at 4°C for 16 h in a Tst 41.14 swinging bucket rotor (Ivan Sorvall, Inc.). The yellow-orange bands in the 30% sucrose layer were removed from the gradients and were pooled. After dilution with 2 volumes of 10 mM TES-sodium hydroxide (pH 7.0)–100 mM NaCl, the membranes were pelleted by centrifugation at 165,000 × *g* at 4°C for 2 h in a Ti50 fixed-angle rotor (Beckman Instruments, Inc.).

For the preparation of total soluble protein, cells (usually 50 ml of culture at 10^8 cells per ml) were harvested by centrifugation at 3,500 × *g* for 5 min at room temperature. The pellet was suspended in 3 ml of 2 mM HEPES (pH 8.0)–100 mM NaCl, with 1 mM benzamidine hydrochloride, 1 mM ε-amino-caproic acid, and 1 mM phenylmethylsulfonyl fluoride. In some cases, the NaCl was omitted to allow complete dissociation of the phycobilisomes. This suspension was passed through a French pressure cell at 100 MPa and centrifuged at 3,000 × *g* for 10 min at 4°C to remove unbroken cells; the supernatant was made 100 mM in NaCl (if prepared in low salt) and centrifuged for 1 h at 100,000 × *g* in a Ti50 fixed-angle rotor. Soluble proteins were precipitated by making the supernatant 10% in trichloroacetic acid and incubating the solution on ice for 30 min. Precipitated protein was pelleted by centrifugation at 12,000 × *g* for 10 min at 4°C, washed once with ice-cold 90% acetone, dried, and prepared for sodium dodecyl sulfate(SDS)-polyacrylamide gel electrophoresis as described below. Pellets obtained after ultracentrifugation were used as the total membrane fractions.

Protein analysis. Protein and membrane pellets were resuspended by sonication in 0.1 M Na_2CO_3–0.1 M dithiothreitol to a concentration of between 1 and 5 mg of protein per ml. The suspensions were made 1.7% in SDS by adding 0.5 volume of SDS-sucrose (5% SDS, 30% sucrose, and 0.1% bromophenol blue) and were boiled for 1 min. The polypeptides were resolved by electrophoresis on SDS-polyacrylamide gels (7.5 to 15% linear polyacrylamide gradients) by using the Laemmli buffer system (10) and were visualized by staining with Coomassie brilliant blue G-250.

RESULTS

The time course of sulfate uptake at 2 μM $SO_4{}^{2-}$ for unstarved cells and cells deprived of sulfur for 24 h is shown in Fig. 1. Uptake by unstarved cells was linear for the entire course of the experiment (20 min), whereas uptake by starved cells began to decline after about 5 min to a rate approximating that of unstarved cells. This decline in rate was not the result of substrate depletion; by the end of each experiment the cells had consumed, at most, only 5% of the supplied substrate. Utkilen and co-workers (17) observed that cells that had been deprived of sulfur for 12 h had lower internal sulfate pools than unstarved cells and that the levels rose rapidly when sulfate was resupplied. Therefore, one possibility is that sulfate uptake declines once internal sulfate pools are replenished. In subsequent experiments, the initial rates were determined from three or four time points within the first 90 s after the addition of sulfate.

At 2 μM $SO_4{}^{2-}$, the initial rate of uptake by starved cells was 12-fold higher than that of unstarved cells (Fig. 1). To determine the overall kinetics of uptake for starved and unstarved cells, we examined the dependence of initial uptake rates on substrate concentration. The overall affinity of the cells for sulfate was essentially unchanged after 24 h of sulfur starvation (Fig. 2). As calculated from the double-

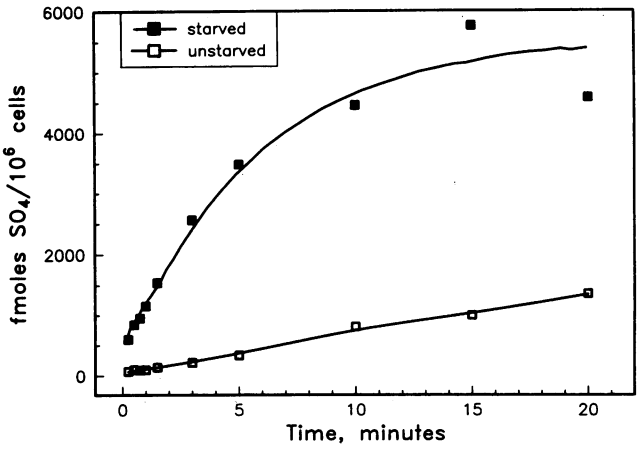

FIG. 1. Time course of $SO_4{}^{2-}$ uptake by unstarved and sulfur-starved (for 24 h) cells. Starved and unstarved cells were transferred to fresh, sulfur-free BG-11 medium to a concentration of 1×10^7 to 5×10^7 cells per ml, and for each assay, 2 ml of the cell suspension was used (see Materials and Methods for incubation conditions). Labeled sulfate was added to 2 μM (specific activity, 750 Ci/mol), and samples were taken for counting at the appropriate time intervals. Each point represents the average of three separate experiments with replicates at each time point.

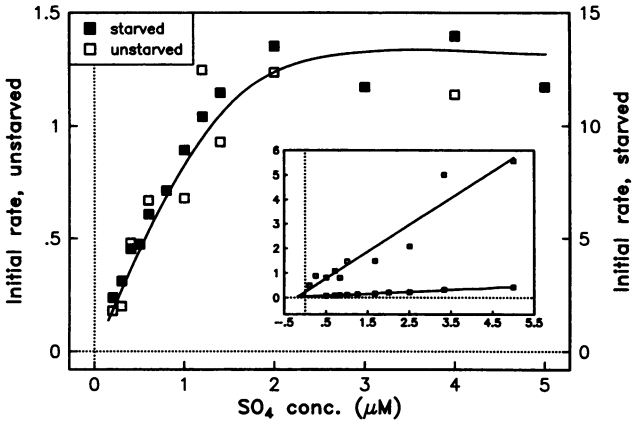

FIG. 2. Dependence of sulfate uptake on external sulfate concentration. Unstarved and sulfur-starved (for 24 h) cells were harvested and suspended in fresh, sulfur-free BG-11 medium to a concentration of 1×10^7 to 5×10^7 cells per ml. The initial rate of uptake (femtomoles/10^6 cells per second) is plotted as a function of external sulfate concentration. Each point is the average of four to six rate determinations. Note the different *y*-axis scales for the two cell types. The inset shows the same data in a double-reciprocal plot. These data predict a $K_{1/2}$ of 1.1 μM and a V_{max} of 1.75 fmol/10^6 cells per s for unstarved cells and a $K_{1/2}$ of 1.6 μM and a V_{max} of 20 fmol/10^6 cells per s for starved cells.

reciprocal plot, the apparent $K_{1/2}$ was approximately 1.1 μM for unstarved cells and 1.6 μM for starved cells. The calculated V_{max} for uptake, however, was increased over 10-fold, from 1.75 fmol/10^6 cells per s for unstarved cells to 20 fmol/10^6 cells per s for starved cells. The rise in the initial rate of sulfate uptake with time after transfer of *A. nidulans* to sulfur-deficient medium showed a lag of approximately 2 h (Fig. 3). The rate of uptake rose sharply between 2 and 6 h and then more gradually up to 24 h after transfer to sulfur-deficient medium. No further increase in rate was observed with longer periods of starvation (data not shown).

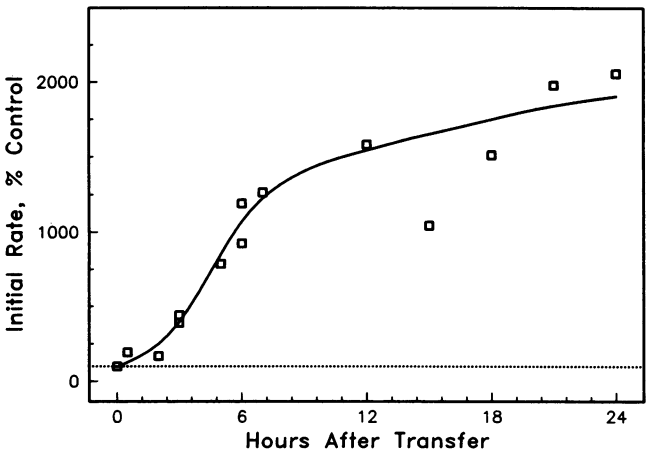

FIG. 3. Increase in initial rate of sulfate uptake after transfer to sulfur-free medium. The data were accumulated from five separate batches of cells which were transferred to sulfur-free medium and assayed for uptake activity at intervals during starvation. Data from four additional batches were pooled for the 24-h time point. The initial rate of sulfate uptake at 1 μM SO_4^{2-} was determined at each time point and was normalized to the control initial rate for cells from that same batch assayed at the time of transfer. The 12-, 18-, and 24-h time points are averages of four, three, and eight determinations, respectively. All other points are single determinations.

Since previous studies suggested that sulfate uptake by *A. nidulans* is an active process, possibly involving a membrane component, we examined the cytoplasmic membrane protein composition during sulfur starvation. There were increases in the levels of five different polypeptides following transfer of cells to sulfate-deficient medium (Fig. 4). Elevated levels of polypeptides with molecular masses of 43, 42, 36, and 33.5 kilodaltons (kDa) were observed within the first 6 h of acclimation to sulfur-limiting conditions, and these levels remained unchanged for at least 24 h. The 36-kDa polypeptide accumulated to the highest levels. As with the rise in initial rate of sulfate uptake, the increase in the abundance of these polypeptides leveled off at 6 to 10 h after transfer to sulfur-deficient medium. A fifth polypeptide (17 kDa) did not appear until the cells had been starved for at least 24 h. The 33.5-kDa polypeptide probably represents a soluble protein which contaminates the cytoplasmic membrane preparation. This polypeptide accumulated to high levels in the cytoplasm of the cells during sulfur starvation and appeared to adhere to all of the membrane fractions of the cells.

To determine whether increases in the initial rates of sulfate uptake and in the levels of the cytoplasmic membrane

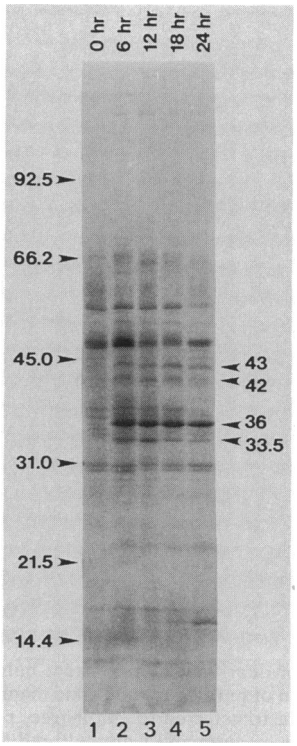

FIG. 4. Effect of sulfur starvation on the polypeptide composition of purified cytoplasmic membranes. Cells were transferred to sulfur-free BG-11 medium and grown for 0, 6, 12, 18, or 24 h. Cytoplasmic membranes were purified from each batch of cells, and the proteins were analyzed by SDS-polyacrylamide gel electrophoresis. The molecular mass standards, indicated by arrows on the left, are as follows: phosphorylase *b*, 92.5 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; bovine carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa. The arrows on the right indicate polypeptides discussed in the text.

proteins are specific to sulfate deprivation and not just part of a generalized stress response, we measured transport by, and isolated cytoplasmic membranes from, cells deprived of nitrogen and phosphorus. After 24 h of starvation for these nutrients, the cells had begun to bleach. While sulfur-starved cells exhibited a greater-than-10-fold increase in their initial rate of sulfate uptake, uptake by nitrogen- or phosphorus-starved cells was reduced relative to unstarved cells. The initial rate of sulfate uptake (in femtomoles per 10^6 cells per second), assayed at 1 μM SO_4^{2-}, was 0.67 ± 0.11 for control cells (n = 5), 8.94 ± 1.85 for sulfur-starved cells (n = 4), 0.20 ± 0.06 for nitrogen-starved cells (n = 3), and 0.13 ± 0.11 for phosphorus-starved cells (n = 3). The 36-, 42-, and 43-kDa polypeptides, present in cytoplasmic membranes from sulfur-starved cells, did not accumulate in cells deprived of either phosphorus or nitrogen (Fig. 5, lanes 1 to 4). The 33.5-kDa polypeptide migrated close to a polypeptide which increased in phosphorus-starved but not in nitrogen-starved cells. However, antibodies against the 33.5-kDa polypeptide from sulfur-deprived cells did not cross-react with any components of total membranes or with the soluble protein fraction from phosphorus-starved cells (unpublished data). A second polypeptide with a molecular mass of 37.5 kDa also accumulated in the cytoplasmic membranes of phosphorus-starved cells.

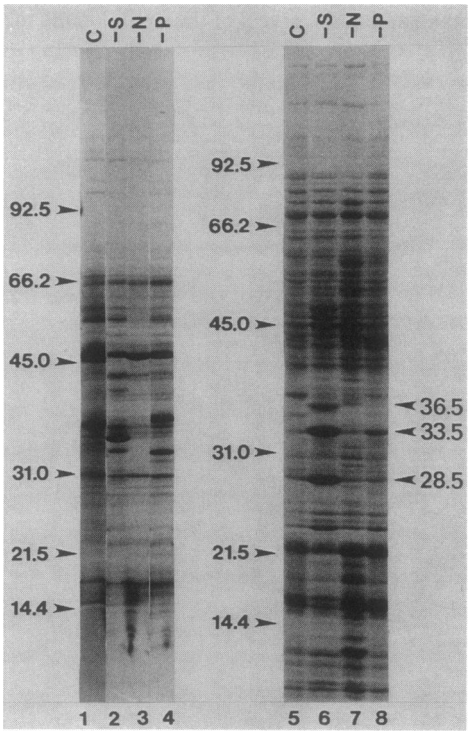

FIG. 5. Effect of deprivation of different nutrients on the polypeptide composition of purified cytoplasmic membranes and soluble proteins. Cells were transferred to sulfur-free, phosphorus-free, or low-nitrogen BG-11 medium and were cultured for an additional 24 h. Cytoplasmic membranes and total soluble proteins were purified from the cultures, and the polypeptides were extracted and separated by SDS-polyacrylamide gel electrophoresis. Polypeptide profiles of cytoplasmic membranes from control (unstarved) cells and sulfur-, nitrogen-, and phosphorus-starved cells are shown in lanes 1, 2, 3, and 4, respectively. Polypeptide profiles of soluble fractions from control cells and sulfur-, nitrogen-, and phosphorus-starved cells are shown in lanes 5, 6, 7, and 8, respectively. The arrows on the right indicate soluble polypeptides discussed in the text. Molecular mass standards, indicated by arrows on the left, are as described in the legend to Fig. 4.

The soluble polypeptides isolated from unstarved cells and from cells deprived of sulfur, nitrogen, or phosphorus are shown in Fig. 5 (lanes 5 to 8). Polypeptides of 36.5, 33.5, and 28.5 kDa (arrows) accumulated to very high levels in sulfur-starved but not in nitrogen-starved or phosphorus-starved cells. A soluble polypeptide which migrated to a position similar to that of the 33.5-kDa polypeptide was present in phosphorus-starved cells; however, as previously mentioned, this polypeptide was not immunologically related to the species present in sulfur-starved cells. The 36.5-kDa polypeptide sometimes separated into a doublet. Other minor changes in soluble polypeptides among the nutrient-stressed and nutrient-replete cells were not consistently observed.

DISCUSSION

When cells of *A. nidulans* were deprived of sulfate, they began to acclimate to the new growth conditions. Among other changes, there was a dramatic increase in their capacity to take up exogenous sulfate, as determined by whole-cell uptake assays. The apparent V_{max} for transport rose about 10-fold in the first 24 h after transfer to sulfur-deficient medium, while the apparent $K_{1/2}$ for sulfate remained constant at about 1.35 μM. These data demonstrate that no new, higher-affinity transport system is induced in response to sulfate deprivation. Therefore, if sulfate transport capacity is regulated at the level of transporter abundance, the regulation is through increased levels of components already present in unstarved cells or through the synthesis of a new transport system with similar characteristics. This strategy may be similar to the one used by *S. typhimurium* (3). It also resembles the regulation of phosphate transport by *A. nidulans* in response to phosphorus deprivation (4).

Examination of the cytoplasmic membrane protein profile after the culture medium was depleted of sulfur revealed at least five polypeptides which increase in abundance. Three of these (43, 42, and 36 kDa) may be present at low levels in unstarved cells and reach maximum abundance by about 6 h after transfer to sulfur-deficient medium. A 33.5-kDa polypeptide, present in both soluble and membrane fractions and undetected in unstarved cells, increased with kinetics similar to those of the cytoplasmic membrane polypeptides. A fifth polypeptide (17 kDa) appeared to a variable extent after a longer lag period. All five polypeptides were specifically upregulated in response to sulfur starvation and did not accumulate during nitrogen or phosphorus starvation. Based on these data, we suggest that one or more of the polypeptides which increased in the cytoplasmic membrane may be responsible for the elevated sulfate transport measured in sulfur-starved cells.

Examination of the total soluble protein profiles revealed several interesting changes. As previously reported (15, 18), there is a decrease in the phycobiliprotein content in sulfur-starved cells. Concomitant with the loss of phycobiliproteins was the dramatic increase of three soluble polypeptides. High levels of accumulation of these polypeptides were restricted to sulfur-starved cells; they were not seen when cells were deprived of nitrogen or phosphorus. Likewise, they did not match soluble proteins which appear with acclimation of *A. nidulans* to low CO_2 conditions (13) or in response to heat shock (12). The specificity of the induction of these polypeptides and the high levels to which they were synthesized argues against their involvement in a general

stress response. They could be enzymes specific to the sulfate assimilation pathway which, as in *S. typhimurium* (9), might be upregulated in concert with sulfate transport. However, their abundance suggests that they probably do not have a catalytic function. They could also be structural components associated with polyphosphate granules or other inclusion bodies which accumulate during sulfur starvation, although many of these inclusion bodies also accumulate in response to other nutrient stresses. Finally, they could be involved in starvation-induced dormancy or in maintaining viability during prolonged sulfur deprivation. We are using a molecular genetic approach to test some of these possibilities.

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