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Nutritional control of copper uptake in the cyanobacterium *Nostoc calcicola* Bréb

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Summary. The kinetics of Cu uptake in nutritionally starved cells of the diazotrophic cyanobacterium *Nostoc calcicola* Bréb. have been compared with those in cells recovering from starvation. Unstarved cyanobacterial cells assimilated 97.0 nmol Cu mg^{-1} protein within 1 h when incubated in medium containing 40 μM Cu. Uptake was markedly inhibited in carbon-starved cells and, to a lesser extent, in cells starved of nitrogen or sulphur. The intracellular concentrations of protein and photopigments were markedly lower in cells starved of carbon, nitrogen, sulphur or phosphorus, whilst that of carbohydrate was lower in cells starved of carbon, sulphur or phosphorus, but almost doubled in cells starved of nitrogen. The ability to assimilate Cu was partially restored in cells after 72 h of recovery from phosphorus or sulphur deprivation, but showed little improvement during recovery from carbon or nitrogen starvation. A possible role of phosphorus in regulating Cu transport and accumulation is discussed.

Key words: Cu uptake - Starvations - Recovery - ATP - *Nostoc calcicola*

Introduction

Manipulation of elemental composition of nutrient medium can generate valuable information covering the morphology, physiology and ultrastructure of cyanobacteria (Sicko-Goad and Jensen 1976; Lawry and Jensen 1979). For example, the nutritional status of cyanobacteria can affect photosynthetic pigments (Jensen and Sicko 1974; Yamanaka and Glazer 1980; Wood et al. 1986), synthesis and utilization of reserve products like cyanophycin during nitrogen starvation or excess (Fay 1983) or polyphosphate under conditions of phosphate excess or limitations (Bone 1971; Healey 1973; Ihlenfeldt and Gibson 1975). Investigations of ultrastructure have suggested that the nutritional status of the organism may affect its susceptibility to environmental pollutants such as heavy metals (Jensen and Rachlin 1984). While most studies involving metals and cyanobacteria have been directed at investigating nutrient uptake and assimilation (Singh and Yadava 1983, 1984; Singh et al. 1987; Pettersson et al. 1988), only a few have been extended to account for the factors that might control metal uptake (Singh and Yadava 1985; Pettersson et al. 1986; Campbell and Smith 1986). The present investigation reports the effect of nutrient status on copper uptake by the cyanobacterium *Nostoc calcicola* Bréb.

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Materials and methods

Organism and growth conditions. The diazotrophic cyanobacterium *Nostoc calcicola* Bréb. was a local isolate from the rice field. Axenic clonal populations were grown photoautotrophically in a modified nitrogen-free inorganic medium (Allen and Arnon 1955) containing A_6 trace elements devoid of copper. Cultures were illuminated with cool daylight fluorescent tubes (14.4 Wm^{-2}) under a 16-h light/8-h dark cycle at 24±1° C unless otherwise specified.

Nutrient deficiency. To induce nutrient deficiency, the 6-d-old cyanobacterial culture was incubated for 72 h in the similar growth medium lacking the respective ions. $MgCl_2$ (1.0 mM) replaced $MgSO_4$ during sulphur starvation and KCl (2.0 mM) replaced K_2HPO_4 in phosphorus starvation. Nitrogen-deficient cells were obtained by growth under argon + CO_2 (95:5, by vol.). For carbon starvation, cultures of the same age were incubated in the dark for 72 h. During this treatment, cultures appeared to have metabolized all their cellular carbon reserve.

In order to assess any correlation between recovery of cyanobacterial cells from nutrient deficiency and their ability to assimilate Cu, K_2HPO_4 (2.0 mM) or $MgSO_4$ (1.0 mM) was added to the growth medium containing phosphorus- or sulphur-deficient cells. Similarly, nitrogen-starved cells were transferred to air, while carbon-starved cells were illuminated. Such cultures were again allowed to recover for 72 h prior to screening for Cu uptake.

ATP measurements. Extraction of ATP with perchloric acid ($HClO_4$) was carried out as described by Larsson and Olsson (1979): 1 ml 1.4 M $HClO_4$ and 0.2 ml 10 mM EDTA were simultaneously injected to 1-ml algal samples containing control, nutri-

tionally depleted or recovered cells followed by a 25-min incubation at 0° C. A mixture of 2.5 M KOH and 0.2 M Hepes was added to neutralize the sample. After mild centrifugation and dilution (1:10) with 20 mM Tris, 2 mM EDTA pH 7.5, the samples were stored frozen (−20° C) for a maximum period of 2 weeks.

Assay of ATP content was conducted according to Pettersson and Bergman (1989) using the firefly luciferin/luciferase/ATP monitoring reagent, which produced a constant light signal proportional to ATP concentration in the samples. Fresh or thawed samples were further diluted (1:10) with Tris/EDTA buffer, a 100-µl extract was put into the sample cuvette with 100 µl Tris/EDTA buffer and 200 µl reagent. ATP/Na_2H_2 was used as the internal standard. The relationship between the light signal produced and the concentration of ATP was found to be linear between 0.05–50 nM ATP in an F 3010 fluorescence spectrophotometer (Hitachi). Values are expressed as molar amounts/mass of chlorophyll *a*.

Cu uptake experiments. Exponential cultures of *N. calcicola* (6-d old) were centrifuged (3000 rpm), washed three times with triple-glass-distilled water and suspended in 10 mM phosphate pH 7.0 to a final concentration of 0.4 mg protein ml^{-1}. Uptake of Cu was monitored in the presence of 2.5–50 µM $CuSO_4 \cdot 5H_2O$ (BDH, India). For analysing Cu content, a 10.0-ml volume of cell suspension was removed at regular time intervals, centrifuged (3000 rpm; 75 s) and the cell pellet washed with EDTA (10.0 µM) to remove the adsorbed Cu. The washed cells were dried and digested in 1 ml $HNO_3/HClO_4$ mixture (10:1, by vol.) in a boiling water bath for 30 min to ensure digestion and the release of associated Cu ions. After cooling, the samples were diluted to 5.0 ml with triple-glass-distilled water. A further centrifugation removed any undigested material and the resulting supernatant was analysed for Cu (expressed as molar amount/mass protein) by atomic absorption spectrophotometry using a Perkin-Elmer 2380 atomic absorption spectrophotometer at 324.7 nm.

Protein was estimated by the method of Lowry et al. (1951), as modified by Herbert et al. (1971). Lysozyme (Sigma) was used as a protein standard. Total carbohydrate was estimated by the method of Dubois et al. (1956) and photopigments as described by Mackinney (1941).

All data presented are the means of triplicate observations with standard error shown as bars. The data for metal uptake and exposure time were verified for the significance at a particular probability level, and the variance ratio (F) calculated as: F=treatment mean square/residual mean square.

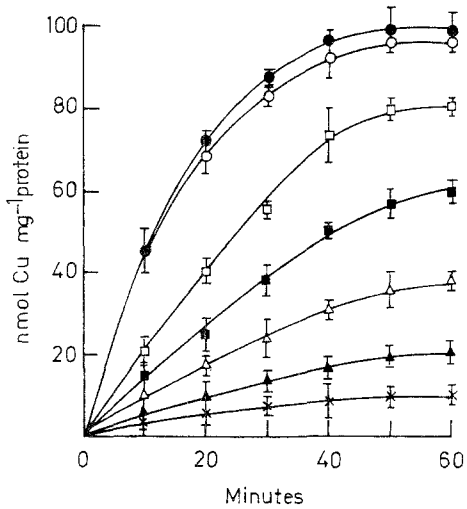

Fig. 1. Cu uptake in *N. calcicola* in relation to varying Cu concentrations.: 2.5 µM (×), 5.0 µM (▲), 10 µM (△), 20.0 µM (■), 30.0 µM (□), 40.0 µM (○) and 50.0 µM Cu (●). $F_{Cu\,6,30}=31.23$, $P<0.01$; $F_{min\,5,30}=12.39$, $P<0.01$

Results

Cu uptake in normal and starved cells

Both the initial rate of Cu uptake and total cellular intake over the period (0–60 min) was dependent on the concentration of Cu supplied in the medium (Fig. 1). Above 40.0 µM Cu, the rate of uptake appeared to be saturated. In subsequent experiments, therefore, 40.0 µM Cu was used. In cells starved of carbon for 72 h, there was a marked decrease in Cu uptake as the initial rate was 11% of that in the nutrient-replete cells. There was a comparatively smaller decline in Cu uptake for cells starved of nitrogen, phosphorus or even sulphur. In phosphorus-starved cells, Cu uptake ceased after 30 min.

Cellular protein, carbohydrate and photopigments

The data on macromolecular levels of the variously starved cyanobacterial cells show that the cellular protein content declined by 82% in sulphur- or nitrogen-starved cells followed by phosphorus- (93%) and carbon-starved (96.7%) cells. In contrast, the total cellular carbohydrate registered an abrupt twofold increase in cells starved of nitrogen over the air-grown control. Such an increase, however, was never recorded in either sulphur-, phosphorus- or carbon-starved cells. While phosphorus-starved cells showed least effect on carbohydrate synthesis (only 40% decrease, compared to control), sulphur and carbon starvation resulted in a significant decline (i.e. 68% and 84%, respectively; Table 1).

The amount of chlorophyll *a* in the test organism also declined in a similar pattern to that observed for protein values with only 43% decrease in nitrogen-starved cells, followed by sulphur- (63%), phosphorus- (78%) and carbon-starved (92%) cells. Phosphorus deficiency, however, resulted in the least decline in the corresponding carotenoid values compared to phycocyanin (80%). Such was not the case in cells suffering either sulphur, nitrogen or carbon starvation for a 72-h span.

Nutrient recovery

Phosphorus recovery of starved cells showed an improvement in Cu intake rate (2.0 nmol mg^{-1} protein min^{-1}), as well as in the increase (20%) in total intracellular Cu concentration over the phosphorus-starved sets (compare Figs. 2 and 3). Sulphur and carbon starvation, which caused widely different Cu intake values, were very similar in their recoveries with an average improvement of 5.52%. Nitrogen recovery, however, showed a negligible improvement as there was only a 2.0% increase in Cu intake over the starved cells. The overall sequence of Cu intake in the 'recovered' cells could be arranged as $P>S=C>N$, thus emphasizing the vital role of phosphorus.

Table 1. Variation in levels of cellular protein, carbohydrate and photopigments in *N. calcicola* following nitrogen, sulphur, phosphorus or carbon starvation (72 h in each case)

Parameter	Value (μg ml^{-1}) for cells grown				
	in normal medium	−N	−S	−P	−C
Protein	875.6±18.608	175.50± 8.68	157.50±10.23	61.25±6.34	11.23±2.034
Carbohydrate	280.0± 7.329	544.40±13.24	89.60± 7.32	168.00±8.66	44.80±2.68
Chlorophyll *a*	12.0± 2.301	6.84± 1.36	4.44± 0.23	2.64±0.143	0.96±0.233
Carotenoid	8.0± 2.103	1.33± 0.86	1.60± 0.177	4.66±0.366	0.66±0.176
Phycocyanin	135.2±12.509	1.35± 0.68	29.24± 3.43	29.24±2.34	0.66±0.176

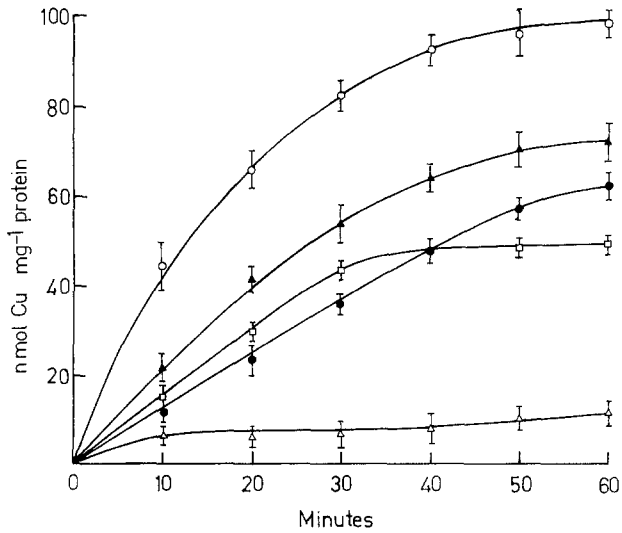

Fig. 2. Comparison of Cu uptake pattern in normal *N. calcicola* cells grown photoautotrophically in the basal medium (○) and those starved of sulphur (▲), nitrogen (●), phosphorus (□) or carbon (△) (72 h each). $F_{\text{starvation } 4,20} = 60.23$, $P < 0.005$; $F_{\text{min } 5,20} = 15.81$, $P < 0.005$

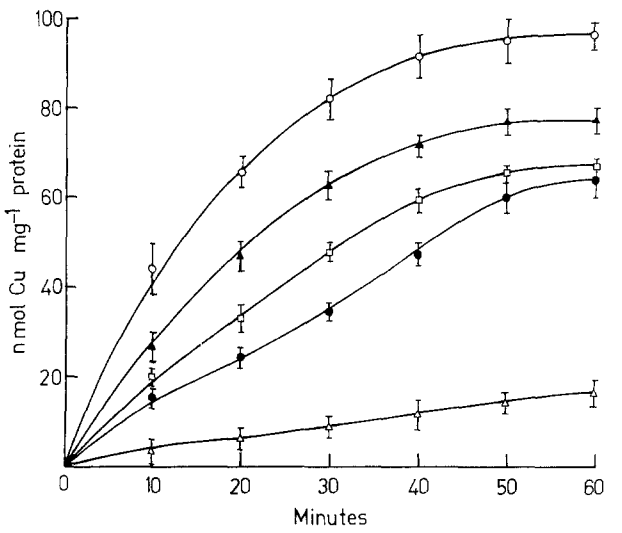

Fig. 3. Cu uptake in normal *N. calcicola* cells (○) and those recovering from sulphur (▲), phosphorus (□), nitrogen (●) or carbon (△) starvation (recovery time, 72 h in each case). $F_{\text{recovery } 4,20} = 72.33$, $P < 0.001$; $F_{\text{min } 5,20} = 24.34$, $P < 0.001$

Table 2. A comparison of ATP pool in normal *N. calcicola* cells under nitrogen, carbon, phosphorus and sulphur starvation (72 h) and after recovery (72 h)

Treatment	ATP pool (nmol mg^{-1} Chl *a*)
Control	152±3.42
N starvation	86±1.732
C starvation	23±1.32
P starvation	62±3.49
N recovery	94±2.96
C recovery	29±2.69
P recovery	113±4.59
S recovery	129±3.46

ATP content

The data on the variation in the ATP pool caused by changes in the elemental composition of the nutrient medium are incorporated in Table 2. It is apparent that the normal, photoautotrophically grown cells invariably maintained a high ATP pool (152 nmol mg^{-1} chlorophyll *a*); however, cells of the same culture age starved of one of the four elements for a constant duration of 72 h displayed different patterns of cellular ATP pool, with a maximum reduction (84.87%) in carbon-starved 'dark cells' and a minimum (26.32%) for sulphur-starved cells, with intermediate values for phosphorus (59.3%) and nitrogen (43.42%) starvation. Cells recovering from starvation, however, exhibited a different pattern with a maximum recovery of cellular ATP pools (33.7%) in phosphorus-supplemented cells followed by sulphur (11.18%), nitrogen (5.22%) and the least (3.95%) during carbon recovery.

Discussion

Metal bioaccumulation in microorganisms is a metabolism-dependent, energy-requiring process (Norris and Kelly 1977; Gipps and Coller 1980; Khummongkol et al. 1982; Singh and Verma 1988). The present study has also shown that *N. calcicola* cells removed Cu ions from the medium in two distinct phases: the first involves a rapid binding/uptake of the cation (within the first 10 min of incubation), followed by the second phase of much lower uptake rate for at least 1 h. Data

in Fig. 1 clearly reveal that Cu uptake by the algal cells was dependent on the amount of Cu present in the ambient medium, as well as on the length of metal exposure. Cu-uptake kinetics showed a hyperbolic nature, saturating at 40 μM Cu. Similar observations also exist for Zn, Cd or Cu uptake in *Anacystis nidulans* (Shehata and Whitton 1982; Singh and Yadava 1985; Singh 1985).

A survey of the available literature indicates very few reports on specific metal transport systems in cyanobacteria. Similar experiments involving Cd revealed that metal uptake also followed Michaelis-Menten kinetics with a K_m of 4.4 μM and a V_{max} 0.83 nmol mg^{-1} protein h^{-1} in *A. nidulans* (Singh and Yadava 1985). A comparison of Cu uptake by normal and starved cells shows a marked reduction in metal intake in the latter case; 72 h of sulphur starvation resulted in a higher rate of metal intake (2.03 nmol Cu mg^{-1} protein min^{-1}) than cells suffering nitrogen, phosphorus or carbon starvation (Figs. 2 and 3). Since sulphur deficiency causes increased phosphate intake in cyanobacteria (Lawry and Jensen 1979), and since polyphosphates are the main metal-accumulation centres (Pettersson et al. 1985), we attribute the enhanced cellular Cu intake/build-up to a larger accumulation of polyphosphate bodies in sulphur-starved cells of *N. calcicola*. Also, sulphur-starved cells did not appear chlorotic as a major portion of the chlorophyll *a* pigment (37% of control cells) remained (Table 1).

The cyanobacterial response to phosphorus deficiency, has been described in terms of elongation of cells in the *Plectonema boryanum* (Jensen and Sicko 1974), elongated trichomes in *Rivularia* sp. (Guerts van Kessel et al. 1977), increased hair formation in several Rivulariaceae (Sinclair and Whitton 1977; Livingstone et al. 1983; Wood et al. 1986), loss of polyphosphate bodies and expansion of thylakoids (Jensen and Sicko 1974; Sinclair and Whitton 1977), loss of gas vacuoles (Reynolds and Walsby 1975), accumulation of cyanophycin granules in *Agmenellum quadruplicatum* (Stevens et al. 1981) and *A. nidulans* (Lehmann and Wöber 1976), apart from physiological changes like decrease in dark-respiration (Senft 1978), rates of nitrogen fixation (Stewart et al. 1970; Bone 1971) and ATP content (Batterton and Van Baalen 1968), in contrast to increase in phosphatase activity (Horichu et al. 1959; Torriani 1960). Therefore, a lowered Cu uptake (1.46 nmol Cu mg^{-1} protein min^{-1}) in *N. calcicola* is attributed to the concomitant lowered availability of ATP pools, while the straight line, symbolizing no further uptake (after 30 min), could be due to non-availability of the metal sink, i.e. polyphosphate bodies, as such cellular phosphate reserves are broken down under phosphorus starvation.

The initial Cu uptake rate in nitrogen-starved cells (0.83 nmol Cu mg^{-1} protein min^{-1}) was invariably lower than in either phosphorus- or sulphur-starved cells. However, unlike phosphorus starvation, Cu intake subsequent to nitrogen starvation continued rising until the end of the experiment (60 min). A plausible explanation offered for the initially low but prolonged Cu uptake in the latter case could be the altered C/N ratio, because nitrogen deficiency in cyanobacteria results in the loss of phycocyanin (Allen and Smith 1969), depletion of phycobilisomes (Tandeau de Marsac 1978; Wood and Haselkorn 1979), decline in RNA content (Grillo and Gibson 1979), in contrast to increase in glycogen content amounting to 60% of the dry mass (Lehmann and Wöber 1976) and cellular polyphosphate (Canto de Loura et al. 1987). Therefore, slow but continuous Cu intake in nitrogen-starved cells could be attributed to the readily available polyphosphate bodies (due to their high accumulation under nitrogen starvation), the most efficient metal sink as discussed above.

The lowest curve in Fig. 3 with negligible rate of Cu uptake (0.33 nmol Cu mg^{-1} protein min^{-1}) signifies the vital role of carbon in the maintenance, multiplication and survival of cyanobacteria. The possible correlation between carbon and nitrogen assimilation in *A. cylindrica* (Fogg and Than-Tun 1960) or cellular C/N ratio and C/N assimilation ratio (Cobb and Myers 1964) have collectively suggested that the basic carbon skeleton for all biosynthetic reactions becomes limited under conditions of carbon starvation. Additionally, organic carbon, the most preferred reserve material, also becomes no longer available for generating ATP to drive the energy-dependent ion transport for further processes.

A maximum of 20% enhancement in Cu intake for cells recovering from phosphorus starvation signifies the vital role of this element in regulating cellular transport/accumulation of Cu or any essential ion. Low recovery of metal intake for sulphur- and carbon-starved cells, on the other hand, emphasizes their insignificant roles in metal accumulation or, in other words, the cells could not recover fully from these starvations within a stipulated period of 72 h, unlike phosphorus-starved cells. The minimum recovery (2%) for cells recovering from nitrogen starvation suggests that nitrogen metabolism, being vital to the general maintenance of cells, may not be instrumental in regulating Cu uptake.

A comparison of values for Cu uptake and the respective cellular ATP pools for nutritionally starved cells shows almost a positive correlation between the two events (compare Fig. 2 and Table 2). A similar correlation could also be suggested for cells recovering from starvation. Such observations further substantiate our previous report that metal intake is dependent on cellular energy levels (Verma and Singh 1990).

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