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Copper Toxicity and Phosphate Utilization in the Cyanobacterium *Nostoc calcicola*

S. K. Verma,[1] R. K. Singh,[1] and S. P. Singh[2]

[1]School of Life Sciences, University of Hyderabad, Hyderabad 500 134, India and [2]Algal Research Laboratory, Centre of Advance Study in Botany, Banaras Hindu University, Varanasi 221 005, India

Copper is an essential micronutrient required for photoautotrophic growth of cyanobacteria as it is a component of plastocyanin in the photosynthetic electron transport chain. Earlier studies suggested that Cu beyond a threshold level inhibits growth (Singh and Verma 1988), photosynthesis (Singh and Singh 1987) and the key enzymes of nitrogen metabolism (Singh et al. 1987) in diazotrophic cyanobacteria. Investigation of ultrastructure has suggested that the nutritional status of the organism may affect its susceptibility to environmental pollutants such as heavy metals (Jensen and Rachlin 1984). Metal tolerance in many plants is reported to be associated with their efficiency in utilizing phosphate or their ability to sustain phosphate deficiency (Foy et al. 1978) which causes marked reduction in cellular ATP content (Verma et al. 1991), rate of nitrogen fixation (Stewart et al. 1970) and dark respiration in cyanobacteria (Senft 1978). That phosphate plays an important role in regulating cellular uptake of copper, has clearly been established using the nutritionally starved cyanobacterium Nostoc calcicola (Verma et al. 1991), but the effect of Cu on phosphate uptake and utilization has not been studied much. Previous studies on metal-phosphate interactions in cyanobacteria have shown conflicting results, while Cd is reported to inhibit phosphate uptake in Anacystis nidulans (Singh and Yadav 1984), Al retarded only its mobilization in Anabaena cylindrica (Pettersson et al. 1988)

Here we present evidence to show that the Cu toxicity in cyanobacteria is due to the Cu-induced phosphate starvation and that exogenous addition of phosphate can antagonize Cu toxicity in the cyanobacterium Nostoc calcicola.

MATERIALS AND METHODS

The diazotrophic cyanobacterium, Nostoc calcicola, a local isolate from rice fields, was grown in Allen and Arnon's (1955) medium as described earlier (Verma and Singh 1990). For studying the role of phosphate on Cu-induced growth inhibition, exponentially grown cells were resuspended in sterile growth medium supplemented with varying concentrations of potassium phosphate (1-5 mM). Cu(3 μM, LD50 concentration: data not shown) was added as cupric sulphate from freshly prepared stock. In another experiment, phosphate starvation was created

Send reprint requests to S.K. Verma at the above address

by incubation of cyanobacterial cells (72 hr) in growth medium lacking phosphate.

The cellular uptake of Cu by the cyanobacterium was monitored as described by Verma and Singh (1990) using a Perkin-Elmer atomic absorption spectrophotometer, AAS - 2380. Assay of cellular ATP content was conducted according to Pettersson and Bergman (1989). Phosphate uptake experiments were conducted in media supplemented with graded concentrations of phosphate (0.25 - 2.5 mM). Phosphate uptake was measured as the depletion of phosphate from the medium by cyanobacterial cells and these amount was estimated according to the method described in APHA (1976).

Alkaline phosphatase activity in phosphate-deficient (-P) and phosphate-sufficient (+P) cells exposed to different Cu concentrations (5 - 40 µM) was monitored by the method of Ihlenfeldt and Gibson (1975) using para-nitrophenol phosphate (pNPP) as substrate. Photoautotrophic growth was measured as changes in total protein content every 24 hr and expressed as mean mass doubling time (µ) following Kratz and Myers (1955). Protein content was estimated by the method of Lowry et al. (1951).

RESULTS AND DISCUSSION

Most metal studies favor cation action of phosphate uptake in cyanobacteria through competitive or non-competitive inhibition (Singh and Yadav 1984). However, such was not the case with A. cylindrica, as Al, being non-inhibitory to cellular phosphate uptake, affected only the mobilization of polyphosphate bodies (Pettersson et al. 1988).

In the present study, the toxic effect of Cu on N. calcicola was markedly dependent on phosphate concentration in the growth medium. Exogenous addition of phosphate (3.0 mM) significantly increased growth and survival of cells dosed with 3.0 µM Cu (LD50 concentration) compared to lower or even higher phosphate concentrations. Notably, the cells incubated in phosphate-less, Cu-supplemented growth medium showed no increase in absorbance at 650 nm (Table 1). The cellular ATP content which exhibited a 70% reduction by Cu treatment showed almost complete recovery after the addition of 3 mM phosphate. The short-term Cu-uptake experiments under similar phosphate concentrations reflected no change in the cellular build up of copper, suggesting that observed phosphate protection of Cu-induced growth inhibition in N. calcicola is not due to the reduced uptake of the ion, but to immobilization at the cell interior.

If the intracellular phosphate level has a role in regulating Cu sensitivity, it would be reflected in terms of Cu inhibitory effect on phosphate uptake. Thus, we examined the effect of Cu on phosphate uptake by phosphate-deficient (-P) as well as phosphate-sufficient (+P) cells. The -P cells (72 hr starvation) showed correspondingly higher rate of its uptake compared to +P cells. In fact, when compared at the level of 50% inhibition of phosphate uptake, a dose of 15 µM Cu in -P culture was equivalent to 30 µM Cu in +P cultures (Fig. 1). Phosphate uptake by N. calcicola cells in graded concentrations (0.25 - 2.5 mM

Table 1. Effect of exogenous phosphate on mean mass doubling time (hr), cellular Cu uptake (nmol Cu/mg protein) and ATP pool nmol/mg protein) in Cu-less control and Cu-treated cells of Nostoc calcicola. Values are represented as ±3 SE.

Treatment	Doubling Time	Cu-uptake	ATP pool
Control	46	96.69 + 2.31	176.41 ± 5.38
3.0 µM Cu + No phosphate	∞	86.00 ± 2.36	53.52 ± 1.32
3.0 µM Cu + 2 mM phosphate	82	88.40 ± 1.92	126.52 ± 2.41
3.0 µM Cu + 3 mM phosphate	52	95.26 ± 3.42	172.36 ± 4.83
3.0 µM Cu + 4 mM phosphate	60	92.42 ± 1.76	174.52 ± 6.31

phosphate) conformed to the Michaelis-Menten Kinetics (Singh and Yadav 1984). A Lineweaver-Burk plot of phosphate uptake in control sets showed a Km of 1.25 mM and Vmax of 8.33 µM/mg protein/min. The addition of 15 µM Cu (50% inhibitory dose to -P cells brought the Vmax down to 4.52 µM/mg protein/min with the unaltered Km of 1.25 mM. However, 30 µM Cu (50% inhibitory dose for +P culture maintained a Vmax of 2.77 µM/mg protein/min, although the Km in this case too, did not change (Fig. 2). A two-fold difference in Cu concentration inhibiting 50% phosphate uptake under +P and -P conditions suggests that under the former situation, (a) Cu was not effective in regulating the phosphate uptake process, as the cells had enough energy to cope with the metal-stress or (b) most of the Cu ions were rendered immobilized at the cell interior, while in latter, the cells represented an energy-depleted state, and thus, lead to the enhanced Cu sensitivity of the energy-dependent uptake of energy raw material (phosphate). Such observations and the non-competitive inhibition of phosphate by Cu do not suggest the direct interaction between Cu and phosphate, rather than the role of cell energetics in transport/ accumulation of Cu as well as phosphate. This is further reflected by a strong negative correlation between Cu uptake and phosphate uptake process in -P cells ($r = -0.96$, $df = 3$; $p < 0.01$) and +P cells ($r = -0.97$, $df = 3$; $P < 0.01$) with a regression of $\hat{y} = 127.34 - 1.054x$ and $\hat{y} = 106.13 - 2.02x$, respectively (Fig. 3).

Alkaline phosphatase activity has been ideal marker to assess the phosphorus availability and morphological reaction in cyanobacteria (Ihlenfeldt and Gibson 1975). To start with 2-fold highger enzyme activity in -P cells, the extent of inhibition by different Cu dose (5 - 40 µM) was less marked compared to the higher degree of inhibition exercised by smilar Cu concentrations for +P cells. A higher Cu sensitivity of the enzyme is also reflected from the respective 50% inhibitory

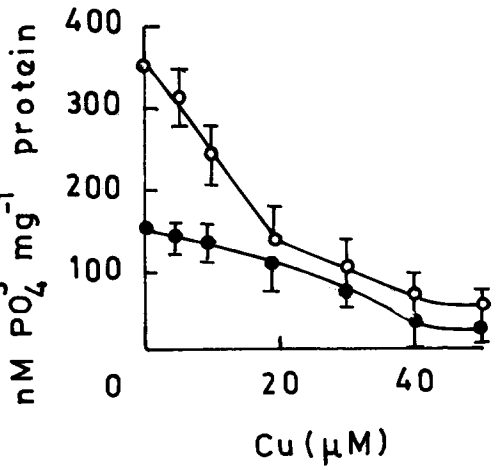

Figure 1. Phosphate uptake in -P (o —— o) and +P (● —— ●) N. calcicola cells supplemented with graded concentrations of Cu.

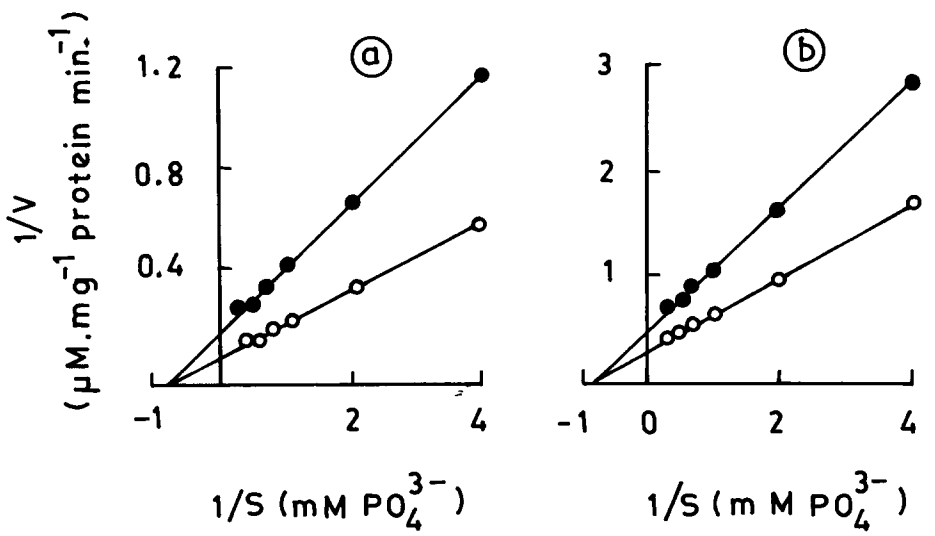

Figure 2. Lineweaver-Burk plots of phosphate uptake at graded concentrations of phosphate in (a) -P and (b) +P cultures; Cu-less control (o —— o) and cells dosed with 50% inhibitory concentrations of Cu (● —— ●).

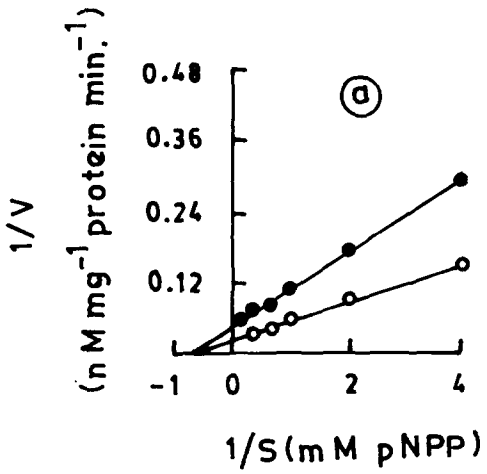

Figure 3. Regression pattern of Cu (X) and phosphate (Y) uptake in -P (o) and +P (●) cells during Cu exposure

Figure 4. Alkaline phosphatase activity in -P (o —— o) and +P (● —— ●) cultures after 1 hr treatment of Cu.

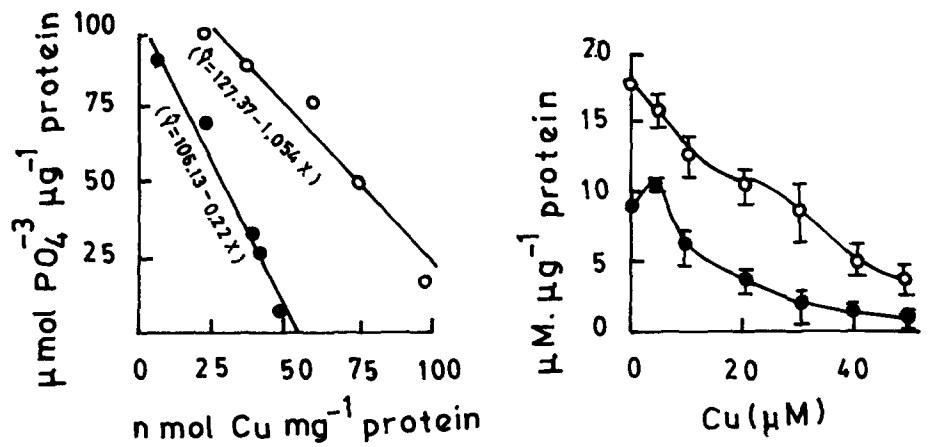

Figure 5. Lineweaver-Burk plots of alkaline phosphatase activity at graded pNPP concentrations in (a) -P and (b) +P cells; control (o —— o) and cells dosed with 50% inhibitory Cu concentration (● —— ●).

concentrations of 32 µM for -P and 15 µM for +P sets. Such comparisons suggest that hypersensitivity of alkaline phosphate in +P cells could be due to lowered synthesis and/or activity of this enzyme compared to induced conditions like -P (Fig. 4). A marked stimulation of alkaline phosphatase activity (27%) by low Cu concentration could be attributed to the activation of enzyme through immobilization of polyphosphate bodies because such cells could have enough cellular phosphate. On the other hand, 10% inhibition of enzyme activity by 5 µM Cu in -P cells possibly suggests that such a cation concentration was available as an inhibitor rather than a requirement or stimulant. The Lineweaver-Burk plot of alkaline phosphatase kinetics under -P condition again reflected a Vmax (36 nmol pNP/mg protein/min) which was 1.5-fold higher than +P cells (25 nmol pNP/mg protein/min), thus supporting reasons for the observed differences in the initial level of enzyme activity. Copper under both conditions significantly lowered the Vmax but did not alter Km (1.20 mM), thus amounting to a non-competitive inhibition of alkaline phosphatase activity. Such inhibitions point towards an indirect involvement of Cu through cellular depletion of energy essential to drive enzyme activity rather than direct competition between metal and enzyme as suggested for Zn (Foy et al. 1978). Similar non-competitive interactions in cyanobacteria have been reported with regard to nitrogenase, glutamine synthetase and nutrient uptake (Stratton et al. 1979; Singh and Yadav 1984), although a direct competition between heavy metal and nutrient ions for uptake has also been reported in a few cases (Singh and Yadav 1983). Therefore, the present pattern of alkaline phosphatase-Cu interaction may not be a rule for other cations or enzymes. As we reported earlier (Verma and Singh 1990), one reason for the sensitivity of cyanobacterium N. calcicola to Cu could be its rapid intracellular accumulation. It is known that cyanobacterial cells grown under +P conditions accumulate heavy metals in polyphosphate granules as the detoxifying mechanism (Jensen and Raclin 1984). In contrast, -P cells apparently would have a reduced capacity to accumulate Cu in polyphosphate bodies, thus -P condition may lead to intracellular Cu being free to act on enzyme(s) and the membrane system.

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