Cu^{2+} TRANSPORT IN THE UNICELLULAR CYANOBACTERIUM *ANACYSTIS NIDULANS*

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Cu^{2+} uptake by *A. nidulans* wild type was concentration dependent and followed Michaelis-Menten type kinetics with saturating concentration at 25 μM . A Lineweaver-Burk plot of the data revealed an apparent K_m of 16.6 μM and a $V_{\max}$ value of 2.5 $\text{nmol mg}^{-1} \text{protein min}^{-1}$. No characteristic pH profile was evident for Cu^{2+} uptake in a pH range of 4.0–9.0, although the highest uptake rate was at pH 4.0. Uptake was sensitive to protonophore FCCP, suggesting the involvement of a proton gradient. Uptake was uninhibited in the dark or in the presence of DCCD, indicating that energy generated through ATP hydrolysis may not be directly involved in the uptake process. Genetic control of the transport was suggested on the ground that mutants differed among themselves as well as from the wild type in terms of K_m and $V_{\max}$ values.

Copper is an essential trace element required in various metabolic processes by microorganisms, algae and higher plants. But once the concentration of Cu^{2+} , as in other heavy metals, reaches a critical level it becomes toxic to the cells. Previous studies on copper toxicity were mostly confined to growth, photosynthesis and nitrogen fixation (1, 2). Recently, copper has been shown to affect the uptake and assimilation of nitrate and ammonium (3, 4).

Copper uptake has been investigated in fresh water algae (5–7), marine diatoms (8), lichens (9) and plant cell cultures (10). It has been shown that algae are capable of accumulating Cu^{2+} to a considerable extent from the surrounding medium (11, 12) against the concentration gradient (13). Several workers have interpreted Cu^{2+} uptake in terms of a multiphasic isotherm (14–17) which may require the existence of a carrier, but which does not necessarily have to be active (18), whereas a biphasic model has been interpreted as an indication of an active process (19). However, two steps have to be understood during the Cu^{2+} uptake study: first, binding of ions on the cell surface and second, uptake of ions across the plasma membrane. So far little effort has been made to study the Cu^{2+} uptake in the cyanobacteria and photosynthetic microorganisms in general, and

no definite relation has been established between copper toxicity and uptake (20).

Keeping this information in view, an attempt has been made (a) to determine the Cu^{2+} uptake rate in the non-nitrogen fixing unicellular cyanobacterium *Anacystis nidulans* and (b) to study the Cu^{2+} transport under the framework of general criteria for transport systems.

MATERIALS AND METHODS

Organism and growth condition. Wild type *Anacystis nidulans* IU 625 (ATCC 27144) was obtained through the courtesy of Dr. R. S. Safferman, Cincinnati, Ohio, USA. The cultures were grown in HUGHES' medium (21), modified by ALLEN (22), supplemented with NaNO_3 (17.6 mM) and A_5 trace elements devoid of Cu^{2+} . The cultures were maintained in a culture room at a temperature of $24 \pm 1^\circ\text{C}$ and illuminated for 14 hr per day by cool day-light fluorescent tubes (intensity approx. 250 ft. c. on the surface of the culture vessel).

Isolation of mutants. Wild type *A. nidulans* (2×10^8 cells ml^{-1}) was exposed to Mn^{2+} (6×10^{-4} M) for 72 hr. The surviving cells (10^8 cells ml^{-1}) were harvested and plated on medium supplemented with 0.5 mM NH_4^+ . Rapidly growing colonies were isolated and tested for resistance to the drug bacitracin. The mutants showing 2–3 fold resistance were selected and numbered *A. nidulans*/Mn⁰, *A. nidulans*/Mn¹⁰ and *A. nidulans*/Mn¹⁴. They were grown in the medium as described for the wild type.

Copper uptake experiments. Exponentially growing cells were harvested and suspended in the buffer (0.01 M, pH 7.0) to a final density of 450 μg protein ml^{-1} . Uptake was initiated by adding Cu^{2+} (2.5 to 25 μM) in the form of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (British Drug Houses, India) to account for the saturating level. The experiment was conducted in light (intensity approx. 250 ft. c.) at a temperature of $24 \pm 1^\circ\text{C}$. Samples were removed at regular intervals of 5–15 min, centrifuged ($4,000 \times g$, 5 min) and analyzed for depletion with respect to time in the Cu^{2+} concentration in the supernatant. The linear portions of the curves were used to determine the uptake rate. The kinetic constants (K_m and $V_{\max}$) were calculated from LINEWEAVER-BURK plots (23).

The pH of the assay medium was adjusted in the range of 4.0 to 9.0 with the following buffers: citrate phosphate buffer (pH 4.0–5.6), sodium phosphate (pH 6.0–7.6) and borate buffer (pH 8.0–9.0). The concentration of the buffer was adjusted to 0.01 M in each case.

Non-specific binding of Cu^{2+} . To determine the non-specific binding of Cu^{2+} adsorbed on the cell surface, cells were suspended in 1.0 mM EDTA at room temperature for 15 min. The cells were separated by centrifugation ($4,000 \times g$, 5 min) and the supernatant was analyzed for copper content.

Inhibitors. N,N'-dicyclohexycarbodiimide (DCCD) and carboxylcyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP) were obtained from Sigma

Chemicals (USA). They were dissolved in ethanol in such a way that the final concentration of ethanol amounted to 0.01% in the assay medium. An inhibitor was added to the assay medium prior to the addition Cu²⁺.

Methods of measurement. Growth was monitored by following changes in optical density ($\lambda=660$ nm). The specific growth rate was calculated by the equation of KRATZ and MYERS (24). Protein was estimated by the method of Folin-phenol (25) using lysozyme as the standard.

Copper concentration was measured colorimetrically as "carbamate" by the method of JACKSON (26).

RESULTS AND DISCUSSION

Effect of Cu²⁺ on growth of wild type A. nidulans and mutants

A comparison of growth of the wild type and mutants in graded concentration of Cu²⁺ is shown in Fig. 1. The effect of Cu²⁺ on growth has been represented in terms of percent increase or decrease in growth rate compared with the control. There was a differential effect of Cu²⁺ on different strains. The 50% growth inhibitory concentration (LD₅₀) of Cu²⁺ was 4 μ M for the wild type, the most sensitive, strain. All the mutants showed resistance to Cu²⁺ in comparison to the wild type: *A. nidulans*/Mn⁹ was 1.25-fold more resistant (LD₅₀=5.2 μ M Cu²⁺), *A. nidulans*/Mn¹⁰ was 2-fold more resistant (LD₅₀=7.4 μ M Cu²⁺) and *A. nidulans*/Mn¹⁴ was 2.5-fold more resistant (LD₅₀=10 μ M Cu²⁺).

Fig. 1. Effect of the graded concentration of Cu²⁺ on the specific growth rate of *A. nidulans* wild type (○), *A. nidulans*/Mn⁹ (●), *A. nidulans*/Mn¹⁰ (⊙) and *A. nidulans*/Mn¹⁴ (+). The results have been expressed as % increase or decrease in growth rate compared with the control (without Cu²⁺).

One of the characteristic feature of *A. nidulans*/Mn¹⁰, unlike other strains, was the apparent stimulation of growth compared with the control, at lower concentrations of Cu²⁺ (1–3 μ M).

Cu²⁺ uptake in wild type A. nidulans

The time course pattern of Cu²⁺ uptake showed that the uptake rate (1.26 nmol mg⁻¹ protein min⁻¹) was a linear function of time for the first 15–20 min of incubation. During the next 15 min, the rate of uptake declined (0.86 nmol mg⁻¹ protein min⁻¹). The uptake rates during the first 15 min of incubation were used in determining the kinetic constants (Fig. 2).

When uptake was studied in different concentrations of Cu²⁺ (2.5–25 μ M), a Michaelis-Menten type of kinetics was observed with saturation in the uptake rate at 25 μ M Cu²⁺ (data not shown). Thus, the uptake process apparently followed a monophasic isotherm in contrast to the multiphasic isotherm reported by WERFF and ERNST (19). It is quite likely that a high affinity transport system might have been missed in the present investigation possibly due to the presence of a trace level contamination of Cu²⁺ in the constituents of the growth medium. This could be the reason for the apparent absence of growth stimulation by Cu²⁺ in the growth experiments. The Lineweaver-Burk plot of the uptake data revealed a K_m of 16.6 μ M and V_{max} value of 2.5 nmol mg⁻¹ protein min⁻¹ (Fig. 4).

pH profile and non specific binding of Cu²⁺ uptake

The Cu²⁺ uptake by *A. nidulans* wild type at different pH levels did not show a characteristic pH profile (Fig. 3). The uptake was maximal at pH 4.0, declined gradually up to pH 7.0, and remained constant between pH 8.0 to 9.0. The pH-

Fig. 2. Time course pattern of Cu²⁺ uptake by *A. nidulans* wild type. Cells harvested during the exponential phase were suspended in citrate-phosphate buffer (0.01 M, pH 4.2) and incubated in light at $24 \pm 1^\circ\text{C}$ temperature. Uptake was initiated by the addition of 25 μ M Cu²⁺.

Fig. 3. Effect of pH on Cu²⁺ uptake by *A. nidulans* wild type (○) and percentage of non-specifically bound (EDTA extractable) Cu²⁺ on the cell surface (●). Different buffers were used to adjust different pH values of the medium (as described in MATERIALS AND METHODS). Uptake was initiated by adding 25 μM Cu²⁺ and was followed for 15 min at each pH value. Samples were withdrawn and centrifuged (4,000 × g, 5 min). The supernatant was analyzed for the total amount of Cu²⁺ taken up by cells in terms of depletion of the Cu²⁺ concentration in the medium. The pellet obtained at each pH was washed with sterilized water and suspended in EDTA (1.0 mM) for 15 min at 25°C. The EDTA solution obtained after centrifugation was analyzed for recovered Cu²⁺ concentration and compared with the total amount of Cu²⁺ taken up by cells in terms of the percentage of EDTA-extractable Cu²⁺ (or non specifically bound Cu²⁺ on cell surface).

dependent Cu²⁺ uptake reflected the involvement of a net charge of ligands in which acidic pH favours the binding of cations. This is in conformity with earlier reports of the decrease of Cu²⁺ toxicity as the pH increases to alkaline range (27).

Since Cu²⁺ binds non-specifically to the cell surface, the extent of binding in the Cu²⁺-fed cells (for 15 min) at each pH was determined by washing the cells with EDTA (1.0 mM) and compared this with the total amount of Cu²⁺ uptake by cells (100%) at corresponding pH values. The results, presented in terms of the EDTA washable fraction as a percentage of the total Cu²⁺ uptake, showed the lowest non specific binding in the acidic range (i.e., 20% at pH 4.0). As the pH was increased, the EDTA washable fraction increased in a linear fashion and reached approximately 85% at pH 9.0 (Fig. 3). Thus, the highest rate of Cu²⁺ uptake was in the acidic range (pH 4.0). The increased toxicity of Cu²⁺ in the acidic range may be attributed to the increase in free Cu²⁺ ions available to the organism (28).

Table 1. Effect of metabolic inhibitors on Cu^{2+} uptake by wild type *A. nidulans*. Cells were suspended in phosphate buffer (pH 7.0, 0.01 M) and inhibitors were added 30 min prior to the addition of Cu^{2+} ($25 \mu\text{M}$).

Inhibitors	Concentration (M)	Cu^{2+} uptake (% of control)
None (control)	—	100
Dark	—	100
DCCD	10^{-5}	100
DCCD	10^{-4}	92
FCCP	10^{-5}	45

Fig. 4. Lineweaver-Burk plots of Cu^{2+} uptake by wild type *A. nidulans* (○), *A. nidulans*/ Mn^9 (●), *A. nidulans*/ Mn^{10} (◐) and *A. nidulans*/ Mn^{14} (+). Experimental conditions were similar to that described in Fig. 3 except that the pH of the assay medium was adjusted to pH 7.0 (Sod. phosphate buffer, 0.01 M).

Table 2. Comparison of kinetic constants of copper uptake in *A. nidulans* wild type and mutants, based on the Lineweaver-Burk plot of the data (Fig. 4).

Strains	Cu^{2+} uptake	
	K_m (μM)	V_{max} ($\text{nmol mg}^{-1} \text{ protein min}^{-1}$)
<i>A. nidulans</i> (wild type)	16.6	2.50
<i>A. nidulans</i> / Mn^9	25	1.42
<i>A. nidulans</i> / Mn^{10}	12.5	2.50
<i>A. nidulans</i> / Mn^{14}	8.3	1.60

Effect of inhibitors on copper uptake

To determine the possible source of energy for Cu²⁺ uptake, the effect of metabolic inhibitors on the uptake was investigated (Table 1). The copper uptake was uninhibited in the dark or in the presence of DCCD, an inhibitor of ATP-ase (29), indicating that light energy or energy generated through ATP hydrolysis may not be directly involved in the uptake process. On the other hand, a protonophore FCCP (30, 31) inhibited the Cu²⁺ uptake to a level of 55%. However, complete inhibition was not observed. This suggests that a proton gradient across the membrane may be governing the Cu²⁺ uptake.

Cu²⁺ uptake in mutants

Since the mutants differed in their sensitivity to Cu²⁺, it was interesting to investigate their relative uptake (Fig. 4). Lineweaver-Burk plots of the uptake data revealed that mutants differed from the wild type regarding affinity as well as uptake rate (Table 2). The mutant *A. nidulans*/Mn⁰ had a lower affinity ($K_m=25\ \mu\text{M}$) and a slower rate of uptake ($V_{\max}=1.42\ \text{nmol mg}^{-1}\ \text{protein min}^{-1}$) than the wild type. On the other hand, the affinity for Cu²⁺ was increased in mutants *A. nidulans*/Mn¹⁰ ($K_m=12.5\ \mu\text{M}$) and *A. nidulans*/Mn¹⁴ ($K_m=8.3\ \mu\text{M}$) with $V_{\max}$ values 2.5 and 1.6 nmol mg⁻¹ protein min⁻¹, respectively. The kinetic constants (K_m and $V_{\max}$) of the mutants differed among themselves as well as from the wild type suggesting a genetic control of Cu²⁺ transport in *A. nidulans*.

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REFERENCES

- 1) E. S. NIELSEN and L. K. NIELSEN, *Physiol. Plant.*, **23**, 828 (1970).
- 2) A. J. HORNE and C. R. GOLDMAN, *Science*, **183**, 409 (1972).
- 3) A. K. KASHYAP and S. L. GUPTA, *Z. Pflanzenphysiol.*, **107**, 289 (1982).
- 4) A. K. KASHYAP and G. JOHAR, *J. Gen. Appl. Microbiol.*, **30**, 267 (1984).
- 5) K. HASSAL, *Physiol. Plant.*, **22**, 304 (1969).
- 6) A. NAKAJIMA, T. HORIKOSHI, and T. SAKAGUCHI, *Agric. Biol. Chem.*, **43**, 1455 (1979).
- 7) P. M. STOKES, *J. Plant Nutr.*, **3**, 667 (1981).
- 8) G. S. CANTERFORD, A. S. BACHANAN, and S. C. DUCKER, *Aust. J. Mar. Fresh Water Res.*, **29**, 613 (1978).
- 9) M. A. S. BURTON, P. LE SUEUR, and K. J. PUCKETT, *Can. J. Bot.*, **59**, 91 (1981).
- 10) W. VELTRUP and F. A. AUSTENFELD, *Plant Cell Reports*, **1**, 31 (1981).
- 11) B. C. RANA and H. D. KUMAR, *Ind. J. Ecol.*, **1**, 1 (1974).
- 12) J. P. RILEY and I. ROTH, *J. Mar. Biol. Assn. U.K.*, **51**, 63 (1971).
- 13) C. E. GIBSON, *J. Appl. Ecol.*, **9**, 513 (1972).
- 14) J. E. BOWEN, *Plant Physiol.*, **44**, 255 (1969).
- 15) W. VELTRUP, *Physiol. Plant.*, **36**, 217 (1976).
- 16) W. VELTRUP, *Z. Pflanzenphysiol.*, **83**, 201 (1977).
- 17) W. G. HARRISON, R. W. EPPLEY, and A. H. RINGER, *Bull. Mar. Sci.*, **27**, 44 (1977).

- 18) N. W. LEPP, *In Effect of Heavy Metal Pollution on Plants*, ed. by N. W. LEPP, Vol. I (1981), p. 111.
- 19) M. M. WERFF and W. H. O. ERNST, *Z. Pflanzenphysiol.*, **92**, 1 (1979).
- 20) G. MIERLE and P. M. STOKES, *In Trace Substances in Environmental Health*, ed. by D. D. Hemphill, University of Missouri, Columbia (1976), p. 113.
- 21) E. D. HUGHES, P. R. GORHAM, and A. ZEHNDER, *Can. J. Microbiol.*, **4**, 225 (1958).
- 22) M. M. ALLEN, *J. Physiol.*, **4**, 1 (1968).
- 23) H. LINEWEAVER and D. BURK, *J. Am. Chem. Soc.*, **56**, 658 (1934).
- 24) W. A. KRATZ and J. MYERS, *Am. J. Bot.*, **42**, 282 (1955).
- 25) O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.*, **193**, 265 (1951).
- 26) M. L. JACKSON, *In Soil Chemical Analysis*, Prentice Hall Inc., N.J. Englewood Cliffs (1958), p. 369.
- 27) E. STEEMAN-NIELSEN, L. KAMP-NIELSEN and S. WIUM-ANDERSON, *Physiol. Plant.*, **22**, 1121 (1969).
- 28) L.P.T.M. ZEVENHUIZEN, J. DOLFING, E.J. ESHUIS and I.J. SCHOLTEN-KOERSELMAN, *Microb. Ecol.*, **5**, 139 (1979).
- 29) H. U. SCHAIRER and B. A. HADDOCK, *Biochem. Biophys. Res. Commun.*, **48**, 545 (1972).
- 30) V. P. SKULACHEV, A. A. SHARAF, and E. A. LIBERMAN, *Nature*, **216**, 718 (1967).
- 31) K. MASAMOTO and M. NISHIMURA, *J. Biochem.*, **82**, 483 (1977).