J. Phycol. **34,** 113–117 (1998)

ROLE OF PROLINE ACCUMULATION IN RESPONSE TO TOXIC COPPER IN *CHLORELLA* SP. (CHLOROPHYCEAE) CELLS[1]

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ABSTRACT

The green alga Chlorella *sp. (strain 2350) was found to accumulate proline, up to a maximum of 0.027 fmol·cell^{-1}, under stressful concentrations of cupric ions. The function of the accumulated proline was studied with respect to its effect on copper (Cu) uptake. By induction with salt stress, cells with various levels of intracellular proline were used for this study. It is shown that the amount of Cu taken up by the cells was reduced when the intracellular proline levels were enhanced. When proline was exogenously supplied prior to Cu treatment, the adsorption of Cu was markedly reduced. When exogenous proline was supplied after Cu treatment, it resulted in a remarkable desorption of the adsorbed Cu immediately after the addition of proline. The results of the present study indicate that proline may exert some action on the cell surface and suggest that one function of accumulated proline is to reduce the uptake of metal ions. The accumulation of proline may be related to a tolerance mechanism for dealing with Cu stress.*

Key index words: Chlorella; *copper; tolerance; uptake; proline accumulation; stress protectant*

Intracellular accumulation of proline is found in a variety of organisms, including algae, bacteria, and higher plants as a response to 1) osmotically stressful conditions such as high salinity, changed osmolarity of growth medium, and drought (Singh et al. 1973, Csonka 1988); 2) temperature shock (Guy 1990); or 3) air pollution (Jäger and Grill 1975, Anbazhagan et al. 1988). The function of proline accumulation seems to be manifold. Proline is considered to play a major role in adjustment to osmotic stresses (Binzel et al. 1987, Ketchum et al. 1991, Voetberg and Sharp 1991). It influences protein solvation (Paleg et al. 1984), stabilizes membranes by interacting with phospholipids (Rudolph et al. 1986), and acts as a cryoprotectant for higher plants (Duncan and Widholm 1987, Songstad et al. 1990, Santarius 1992). Previous studies reported that the accumulation of proline can also be detected in some algal cells during contact with deleterious concentrations of copper (Cu) and cadmium (Wu et al. 1995b). However, the function of proline accumulation in response to metal stress is still unknown.

As the primary target of metal stress, the cell plasmalemma is damaged by Cu (McBrien and Hassall 1965, Barber 1968, De Filippis 1979), leading to the leakage of intracellular potassium ions to the extracellular medium. In previous studies, we have shown that the supply of exogenous proline is effective in reducing potassium leakage from Cu-treated *Anacystis nidulans* cells (Wu et al. 1995a), and, as a result, this interaction might be responsible for reducing the toxicity of Cu. Algae may become tolerant to metal stresses through a variety of mechanisms, such as intracellular binding with metal ions (Rauser 1984, Lee et al. 1996), extracellular exclusion of metal ions (Foster 1977), or a reduction of metal uptake (Singh and Yadava 1986). Nevertheless, it is still unknown whether proline accumulation is related to metal tolerance. Thus, it is of great interest to study the role of proline accumulation in the stressed algal cells. The following study focuses on the effect of proline on the uptake of Cu by the single-celled green alga, *Chlorella* sp.

MATERIALS AND METHODS

Chlorella sp. (strain 2350, isolated from a freshwater pond in Taoyuen county, Taiwan, spherical cells with diameters ranging between 2 and 6 μm) was cultivated in an inorganic culture medium adopted from Kuhl (1962). All cultures were kept at 27° C, aerated with compressed air containing 0.3% CO_2 (flow rate about 60 mL·min^{-1}), illuminated with about 600 μmol photons·m^{-2}·sec^{-1} (with five cool-white fluorescent lamps, F48T12-CW-1500, General Electric) and grown under a light:dark cycle of 14:10 h. Sulfate salts of Cu ($CuSO_4 \cdot 5H_2O$) were added to the cultures at the exponential growth phase.

About 5×10^9 cells were harvested by centrifugation (5000 g × 8 min), resuspended in deionized water (ca. 1.5 mL), and mixed with an equal volume of sea sand. Subsequently, the cells were disrupted with a bead beater (Biospec, Bartlesville, Oklahoma). The cell extracts were then separated from cell debris and sea sand by filtering through a glass fiber filter under reduced pressure. The proline content in the cell extracts was then determined according to the method of Bergman and Loxley (1970).

The increase of cell number was used as a measure of growth rate. The number of cells in the culture was counted by a Coulter counter (Coulter Electronics Ltd., Beds, England). The equation given by Fogg (1975) was employed to estimate the specific growth rate of the cells.

The protein concentration in cell extracts was quantitatively measured by the modified Lowry method (Bollag and Edelstein 1991), using bovine serum albumin (Merck, Darmstadt, Germany) as the standard. The concentration of Cu ions in the medium

[1] Received 7 June 1996. Accepted 10 October 1997.

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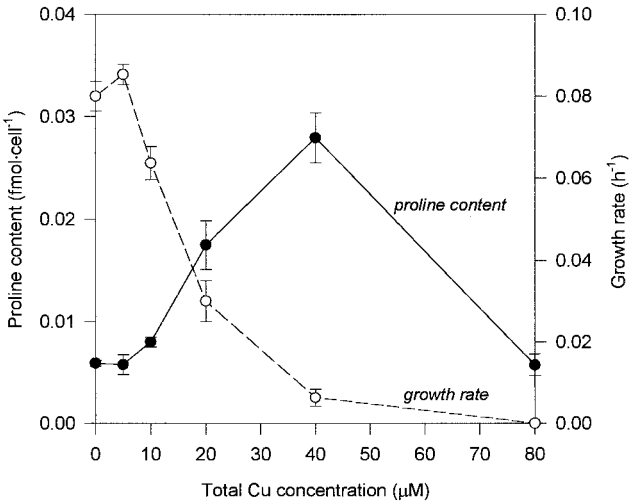

FIG. 1. Changes in the growth rate (unfilled) and intracellular proline content (filled) of *Chlorella* sp. cells as a function of Cu concentrations added. Vertical bars indicate standard error of four independent experiments.

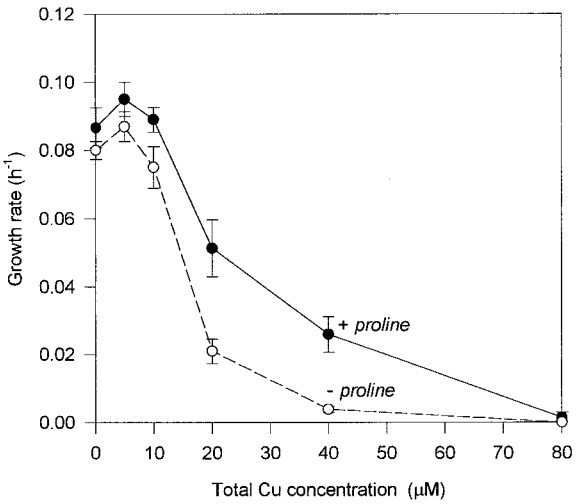

FIG. 2. Dose-response curve of the growth rate of *Chlorella* sp. to Cu concentrations in the presence (filled) and absence (unfilled) of exogenous proline (0.1 mM). Vertical bars indicate standard error of three independent experiments.

was measured with an atomic absorption spectrophotometer (Perkin Elmer 2380, Norwalk, Connecticut). Interfering particles were removed from samples by filtration through a glass fiber filter before measurement. The uptake of Cu by the cells, in terms of cell volume, was calculated on the basis of the average cell volume of 11.49×10^{-12} mL·cell^{-1}. The same was also employed for the calculation of intracellular proline level.

To obtain cells containing various intracellular proline levels, NaCl was used as an inducing factor. Algal cells were first incubated in culture medium containing 0.05%~1.0% additional NaCl, with other culture conditions as noted above. After harvesting the cells by centrifugation to remove extracellular NaCl, cells were resuspended in a NaCl-free culture medium containing 10 μM Cu and incubated under illumination for 1 h. The quantity of Cu removed by the cells was determined and used as a measure of Cu sorption.

To ascertain the toxicity of Cu–proline complex, the concentrations of exogenously supplied proline and Cu (designated as Cu_T) were adjusted, using the complexation constants described by Twiss (1996), so that the free Cu^{2+} ion concentration was kept at 2.5 μM. For these experiments, the proline was added to the culture media prior to the addition of Cu. Subsequently, algal cells were added to give a concentration of $1.8\sim3.8 \times 10^6$ cells·mL^{-1}. The growth rate and the uptake of Cu by the cells were measured after an incubation of 24 h.

All the experiments mentioned above were conducted at least three times, with triplicate measurements for each treatment.

RESULTS

High concentrations of Cu are toxic to *Chlorella* sp. Inhibition of cell growth was observed at concentrations above 10 μM Cu_T (Fig. 1). The cells ceased to grow when exposed to 80 μM Cu_T or higher. During contact with deleterious concentrations of Cu^{2+}, the level of intracellular proline increased as Cu_T increased from 5 to 40 μM, but it was low at 80 μM.

The inhibitory effect of Cu could be lowered, when exogenous proline (optimum concentration 0.1 mM) was supplied to algal cultures (Fig. 2). The effect of proline on lowering the Cu toxicity varied with the Cu concentrations tested. There was no visible lowering in Cu toxicity when the Cu concentration was higher than 80 μM.

Chlorella sp. is rather susceptible to salt stress. Concentration of NaCl as low as 0.05% resulted in a detectable inhibition of cell growth (Fig. 3A). The growth rate of cells decreased with elevating salt concentrations.

Under salt stress, the intracellular proline level was enhanced, with the lowest detectable enhancement at 0.05% NaCl (Fig. 3B). The enhanced proline levels increased with elevating NaCl concentrations from 0.01% to 0.5%. There was no further in-

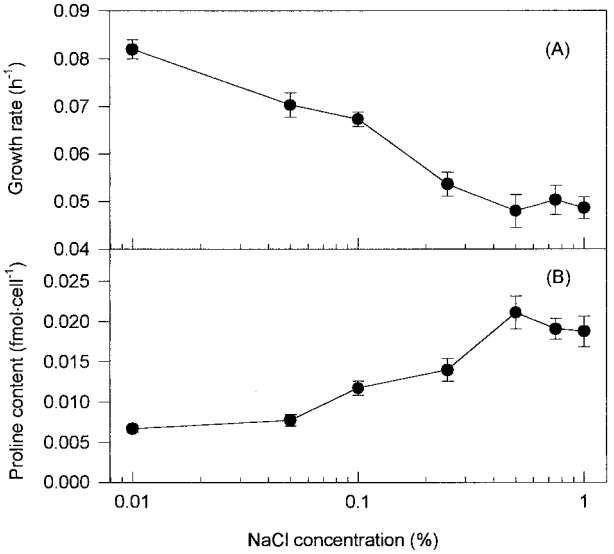

FIG. 3. (A) The growth rate of *Chlorella* sp. cells in the presence of various NaCl concentrations; (B) Content of intracellular proline of the cells grown with various concentrations of NaCl. Vertical bars indicate standard error of three independent experiments.

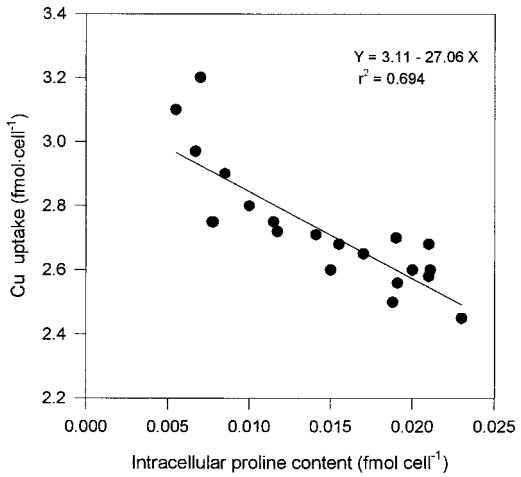

FIG. 4. Plot of the uptake of Cu (at 20 μM) by *Chlorella* sp. cells containing various intracellular proline levels. The cells containing different intracellular proline levels were obtained by pretreating with various NaCl concentrations, which were removed before the measurement of Cu uptake. For detailed procedures, see Materials and Methods.

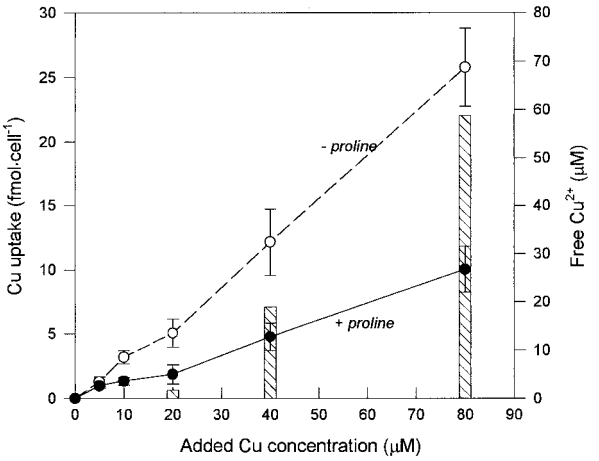

FIG. 5. Effect of the preincubation in the culture medium containing 0.1 mM proline (+proline), compared with the control (−proline), on the uptake of various concentrations of Cu by *Chlorella* sp. cells. Concentrations of free Cu^{2+} (shown by block bar) is calculated by employing the complexation reaction constants given by Twiss (1996). Vertical bars indicate standard error of three independent experiments.

crease in intracellular proline concentration at 0.5%–1.0% NaCl.

When *Chlorella* sp. was pretreated with a variety of concentrations of NaCl, the cells contained different levels of proline. Subsequently, these cells were used for the study of the effect of intracellular proline on Cu uptake. As shown in Figure 4, it is clear that the Cu removed by the cells is negatively correlated with the intracellular proline levels as well as the NaCl concentrations used in the pretreatment.

To study further the effect of proline on Cu uptake, exogenous proline was supplied to the culture medium. The addition of proline (0.1 mM) to algal cultures was made either prior to or after exposure to Cu. When proline was supplied prior to Cu treatment, as shown in Figure 5, the amount of Cu taken up by the cells was markedly reduced when compared with the control. The reduction in Cu uptake was observed either in the medium in which Cu was nearly completely complexed (Cu_T <20 μM) or in those in which Cu was only partially complexed (Cu_T >20 μM) by added proline. These results suggest that some inhibition of Cu uptake is independent of Cu speciation.

When the supply of exogenous proline was conducted after the treatment of Cu, the effect of proline was somewhat different. In the control culture (without the supply of exogenous proline), more than 65% of the added Cu was removed by the cells from the culture medium within 5 min, primarily as a result of the adsorption on cell surfaces. The percentage of sorption did not change markedly in the subsequent time of incubation up to 1 h. When exogenous proline was supplied 10 min after beginning the incubation, as indicated in Figure 6, it immediately caused a large increase in the dissolved Cu concentration in the culture medium, showing a rapid desorption of the adsorbed Cu. In this case, about 80% of the adsorbed Cu was desorbed.

In the following experiments, the concentrations of exogenously supplied proline and Cu (designated as Cu_T) were adjusted, using the complexation constants described by Twiss (1996), so that free Cu ion (designated as Cu^{2+}) concentration was kept constant, as shown in Figure 7. Under such conditions, both the growth rate and the amount of Cu_T taken up by algal cells were not constant with respect to Cu^{2+} concentration. The uptake of Cu was lowered at elevated proline concentrations (Fig. 7A), show-

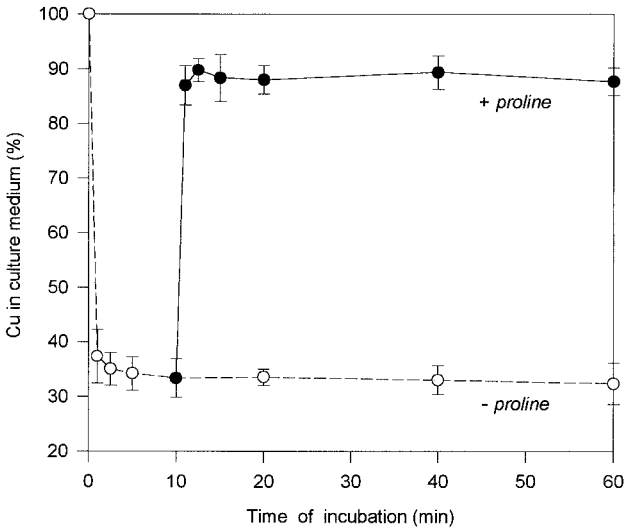

FIG. 6. Effect of the addition (indicated by arrow) of exogenous proline (0.1 mM) to culture medium on the removal of Cu (added Cu_T = 20 μM) by *Chlorella* sp. cells ($1.8\sim3.8 \times 10^7$ cells·ml^{-1}). Vertical bars indicate standard error of four independent experiments.

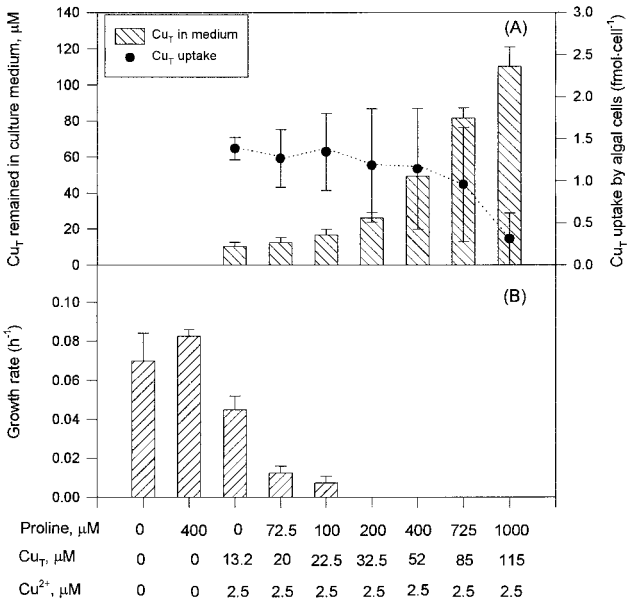

FIG. 7. Effect of various concentrations of exogenously supplied proline on the uptake of added Cu (Cu_T) and the growth rate of *Chlorella* sp. cells. The concentrations of Cu_T and the supplied proline were adjusted so that the concentration of free Cu ion (Cu^{2+}) was kept constant after complexation between them. The sequence of additions of the proline, Cu, and algal cells to the media was given in Material and Methods. Vertical bars indicate standard error of three independent experiments.

ing that proline exhibits an inhibitory effect on the uptake of complexed Cu by algal cells. At Cu_T higher than 22.5 μM, algal cells failed to grow well, regardless of whether the culture medium contained sufficient proline for complexation with Cu (Fig. 7B; cf. Fig. 1). This suggests that the proline–Cu complex was toxic to *Chlorella* sp. cells.

DISCUSSION

In addition to the species reported previously (Wu et al. 1995b), *Chlorella* sp. also accumulates proline intracellularly in response to Cu stress. *Chlorella* sp. cells also accumulate a considerable amount of proline when treated with stressful concentrations of NaCl. The salt-induced enhancement in intracellular proline levels depends on the treated NaCl concentrations. This allows a comparative study of the role of proline in cells containing different intracellular proline levels.

The present study shows that increasing amounts of intracellular proline reduces the internalization of Cu from the external medium by *Chlorella* sp. This could result from either an enhanced efflux of Cu from the cytoplasm or a reduced absorption of Cu. In the former case, it is assumed that the efflux of Cu is enhanced when it is complexed by proline. Our previous study (Wu et al. 1995b) showed that proline accumulation is related to metal tolerance. Regarding the tolerance to metals, a variety of mechanisms have been reported for algae. Most of these mechanisms are related either to a lower uptake caused by altered adsorption (Foster 1977) or to a rapid production of metal-binding proteins, or chelatins, to lower the intracellular free concentration of heavy metal ions (Silverberg et al. 1976). As a strategy of detoxification, chelatin–metal complex can be exported out from the cytoplasm (Lee et al. 1996). Proline has an appreciable affinity to forming various complexes with cupric ions (cf. Martell and Smith 1993, Twiss 1996), which may be partly attributable to the reduction in Cu toxicity. Nevertheless, until now, no published studies have shown that export of metal complex is associated with a reduction in metal uptake by algal cells. Foster (1977) even showed that the tolerant *Chlorella* took up about the same amount of Cu from the bulk solution as intolerant cells but retained more Cu on the cell surface so that fewer metal ions would enter the cytoplasm. Thus, tolerance to Cu is related to the reduced absorption. Our present study shows that the presence of proline reduces the uptake of Cu by *Chlorella* sp. cells. Although no further studies show that the tolerance to Cu of the *Chlorella* strain studied by Foster (1977) is related to proline, the present study and our previous studies suggest that intracellularly accumulated proline is related to the tolerance to Cu in algal cells.

The supply of exogenous proline elucidates the role of proline in *Chlorella* cells. By directly supplying exogenous proline to the algal cultures, the uptake of Cu can be remarkably reduced. In addition, the proline causes a desorption of the adsorbed Cu, detectable immediately after its addition. This effect may be ascribed to the complexation between proline and Cu and the subsequent displacement of the sorption equilibrium between Cu in culture medium and Cu adsorbed on the cell surface.

There are some observations in the present study that cannot be ascribed solely to the rule of a free ion model. For example, algal cells failed to grow at higher Cu_T concentrations (i.e. >32.5 μM), even though Cu has been complexed by proline. This implies that the proline–Cu complex is toxic to *Chlorella* cells, although its toxicity is lower than that of the free ions. Accordingly, the inhibitory effect of Cu on the growth of algal cells in our previous report (Wu et al. 1995b) as well as in the present study should be a combination of the free Cu^{2+} ion and the Cu–proline complex.

It has been ascertained that the cell plasmalemma is the primary target of the toxic action of trace and heavy metals (McBrien and Hassall 1965, Barber 1968, De Filippis 1979). Our previous study with a cyanobacterium, *Anacystis nidulans* (Wu et al. 1995a), showed that proline is capable of inhibiting the leakage of potassium ions, the symptom of damage in the plasmalemma, from Cu-treated cells, suggesting that proline seems to be protective for the plasmalemma. Proline is one of the compatible solutes (Samaras et al. 1994) that have the function of stabilizing the folded protein structure and there-

fore influence protein solvation (Low 1985). Rudolph et al. (1986) assumed that proline may stabilize membranes by interacting with phospholipids. Jolivet et al. (1982) and Zhao et al. (1992) speculated that compatible solutes might protect the membrane integrity and affect the stability of membranes. Parent et al. (1996) showed that fulvic acid, a natural organic ligand, exerts beneficial effects on plasmalemmal permeability of *Chlorella pyrenoidosa*. On the basis of these facts, it is assumed that the physiological role played by intracellularly accumulated proline, in addition to forming complexes with Cu, is possibly to protect the cell membranes by stabilizing plasmalemmal permeability, so that less Cu is taken up by the cells. As a consequence, the cell plasmalemma is damaged less, and less Cu enters the cytoplasm. Accordingly, how proline acts on the cell wall or plasmalemma is worthy of further study.

The authors appreciate the helpful comments and suggestions on part of the experimental designs of this study from Dr. Michael R. Twiss, Woods Hole Oceanographic Institution.

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