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Regulated Copper Uptake in *Chlamydomonas reinhardtii* in Response to Copper Availability[1]

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A saturable and temperature-dependent copper uptake pathway has been identified in *Chlamydomonas reinhardtii*. The uptake system has a high affinity for copper ions (K_m approximately 0.2 μM) and is more active in cells that are adapted to copper deficiency than to cells grown in a medium containing physiological (submicromolar to micromolar) copper ion concentrations. The maximum velocity of copper uptake by copper-deficient cells (169 pmol h^{-1} 10^6 cells^{-1} or 62 ng min^{-1} mg^{-1} chlorophyll) is up to 20-fold greater than that of fully copper-supplemented cells, and the K_m (approximately 2×10^2 nM) is unaffected. Thus, the same uptake system appears to operate in both copper-replete and copper-deficient cells, but its expression or activity must be induced under copper-deficient conditions. A cupric reductase activity is also increased in copper-deficient compared with copper-sufficient cells. The physiological characteristics of the regulation of this cupric reductase are compatible with its involvement in the uptake pathway. Despite the operation of the uptake pathway under both copper-replete and copper-deficient conditions, *C. reinhardtii* cells maintained in fully copper-supplemented cells do not accumulate copper in excess of their metabolic need. These results provide evidence for a homeostatic mechanism for copper metabolism in *C. reinhardtii*.

Copper is an essential micronutrient for all organisms because of its function in enzymes, which serve as catalysts of oxygen chemistry and redox reactions (Frieden, 1985; Linder, 1991). Copper is well suited for its biological function because its ionic states form stable complexes with proteins, are easily interconverted by electron transfer, and the reduced state can be oxidized by oxygen. These very same chemical properties are responsible for the cytotoxicity of copper ions when they accumulate to high concentrations. Copper ion metabolism, therefore, poses an interesting regulatory problem. An organism must maintain an appropriate supply of copper for its use in the biosynthesis of copper enzymes, yet it must prevent the accumulation of excess copper within the cell. Moreover, this must be accomplished in the face of changes in the availability of copper in the extracellular environment.

The importance of maintaining a copper balance is demonstrated by the occurrence of several debilitating diseases in humans and other animals that can result either from copper deficiency or copper toxicity (Owen, 1981). In plants symptoms of copper deficiency and toxicity have been described for a variety of species and natural habitats (e.g. Lonergan et al., 1980; Droppa et al., 1984; Mohanty et al., 1989; Turvey and Grant, 1990; Kabata-Pendias and Pendias, 1992); both types of symptoms have agronomic, and hence economic, consequences. For instance, the accumulation of copper in plants grown on copper-contaminated soils makes the plant unsuitable for use as livestock feed. The consequences of copper deficiency for agricultural crops include sterility and reduced yield, and for forest trees, the loss of marketable wood as a result of limb deformity (Turvey and Grant, 1990). The molecular responses to copper toxicity have been investigated for many organisms, including bacteria, plants, yeasts, and mammals (e.g. Grill et al., 1989; Zhou and Goldsbrough, 1994; reviewed by Rauser, 1990; Steffens, 1990; Brown et al., 1992; Zhou and Thiele, 1993; Solioz et al., 1994). Accordingly, different mechanisms of adaptation to toxicity have been described and are being characterized in ongoing research efforts. Much less attention has been paid to the study of copper deficiency, and thus, the molecular mechanisms underlying adaptation to copper deficiency are not well detailed.

Chlamydomonas reinhardtii serves as a useful model for the study of adaptation to copper deficiency (Merchant et al., 1991). Similar to other green algae, *C. reinhardtii* cells do not appear to contain some of the copper enzymes, such as copper/zinc-superoxide dismutase and polyphenol oxidase, that are abundant in leaves of vascular plants (Lumsden et al., 1977; Sandmann and Boger, 1983; Sherman et al., 1991). Thus, the major copper proteins in this organism are plastocyanin, which functions in the chloroplast in photosynthesis, and Cyt oxidase, which functions in respiration in the mitochondrion. Since plastocyanin is estimated to be at least 10-fold more abundant than Cyt oxidase, the bulk of the copper contained in a C. *reinhardtii* cell can be accounted for by a single protein, plastocyanin. Despite the abundance of plastocyanin (approximately 8×10^6 molecules per cell) and its critical function in oxygenic photo-

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Abbreviations: BCDS, bathocuproine disulfonate; coprogen, coproporphyrinogen; TAP, Tris-acetate-phosphate.

synthesis, *C. reinhardtii*, like other green algae and some cyanobacteria, can survive copper deficiency because of the existence of a copper-responsive regulatory circuit that is involved in the adaptation to copper deficiency (reviewed by Merchant, 1997).

In *C. reinhardtii* the best-studied adaptive response to copper deprivation is the activation of the *CYC6* gene (encoding Cyt c_6, the function at which is equivalent to that of plastocyanin) (Quinn and Merchant, 1995). If the copper content of the medium falls below the amount that is required to support holoplastocyanin accumulation to its usual stoichiometry, transcription of the *CYC6* gene is initiated. The activity of the *CYC6* gene is related to the anticipated plastocyanin deficiency, so that the sum total of plastocyanin plus Cyt c_6 per cell remains constant (Merchant et al., 1991). When the copper content of the medium is sufficient to satisfy the demand of the plastocyanin biosynthetic pathway (approximately 8×10^6 to 9×10^6 copper ions/cell, corresponding to several hundred nanomolar in a typical late log/stationary phase laboratory culture), the *CYC6* gene is not expressed. When the medium is severely depleted of copper (<3 nM), the *CYC6* gene is maximally expressed. The regulatory pathway that controls *CYC6* expression thus functions in response to a range of copper concentrations that correspond to a physiological copper requirement.

In addition to the activation of *CYC6* expression, *C. reinhardtii* cells also induce other processes when they are faced with copper deprivation. This includes the induction of coprogen oxidase (encoded by the *CPX1* gene) and degradation of apoplastocyanin (Hill and Merchant, 1995; Li and Merchant, 1995). Coprogen oxidase induction is rationalized on the basis of an increased metabolic demand for heme as an alternative redox cofactor in Cyt c_6, the substitute protein to plastocyanin, whereas apoplastocyanin degradation is believed to ensure the availability of residual trace copper for other "essential" copper enzymes (such as Cyt oxidase). The *CYC6* and *CPX1* genes of *C. reinhardtii* respond rapidly to supplementation of the medium with as little as 2 to 5 nM copper ions. This suggests that the organism may have an active, high-affinity uptake system under copper-deficient conditions.

In eukaryotic cells copper transport is best characterized in *Saccharomyces cerevisiae*, where copper uptake is believed to involve a cell-surface cupric reductase activity (Hassett and Kosman, 1995) and a high-affinity transport component (encoded by the *CTR1* gene) (Dancis et al., 1994a, 1994b). Expression of the *CTR1* gene is regulated by copper so that it is most active when the medium copper concentrations are low, as is expected for an assimilatory transporter. We sought to characterize the copper uptake pathway of *C. reinhardtii* to determine whether it might be regulated in coordination with *CYC6* and *CPX1* expression during adaptation to copper deficiency. In this work we demonstrate that copper-deficient cells exhibit a greater uptake activity relative to copper-sufficient cells, and we suggest that cupric reductase might be a component of the *C. reinhardtii* copper assimilation pathway.

MATERIALS AND METHODS

Cell Growth

Wild-type cells of *Chlamydomonas reinhardtii* (strain CC 124) were grown under illumination in copper-deficient TAP medium (Merchant and Bogorad, 1986; Harris, 1989). Where indicated, cultures were supplemented with copper sulfate or copper chloride from sterile stock solutions. To induce iron deficiency, cells from iron-supplemented cultures were collected by centrifugation, resuspended in iron-free TAP medium, and returned to the incubator for 6 to 8 h. The preparation and analysis of RNA has been described previously (Merchant and Bogorad, 1986; Hill and Merchant, 1995).

Inductively Coupled Plasma-MS Analysis for Copper

Cells were removed from the medium by centrifugation (5000*g*, 5 min), and the supernatant was acidified by the addition of ultrapure nitric acid (J.T. Baker, Phillipsburg, NJ) to a final concentration of 0.1%. The copper content of the acidified supernatant was measured at the University of Wisconsin Soil and Plant Analysis Laboratory (Madison) by inductively coupled plasma-MS. The detection limit (for copper) is under 1.6 nM. The standards were run in triplicate (SD = ±1%).

Copper Uptake Assay

Copper uptake was measured essentially as described by Lin and Kosman (1990). Cells in log-phase growth were collected by centrifugation (2000*g*, 5 min), washed, and resuspended in uptake buffer (0.2 M Mes, pH 7.0) to approximately equal cell densities. For each sample, the cell density and chlorophyll concentration were determined (average of two to three measurements). Uptake assays were performed at ambient temperature and were initiated by the addition of copper chloride solutions containing ^{64}Cu (University of Missouri River Reactor Facility, Columbia) as a tracer. The cells were well aerated during the assay by shaking at 250 rpm on a platform shaker. To measure the amount of cell-associated ^{64}Cu, samples (0.5–1.0 mL) were diluted into 4.0 mL of an ice-cold "quench" solution (10 mM EDTA, 100 mM Tris-succinate, pH 6.0), filtered by suction through a 0.45-μm nitrocellulose filter (Millipore), and washed with 10 mL of the quench solution. The radioactivity that was associated with the cells was measured in a gamma counter (LKB CompuGamma, Bromma, Sweden). The output, which was corrected for background, counting efficiency, and decay, was used to estimate the amount of cell-associated copper. For determination of the initial velocity of uptake, the samples were collected during the linear part of the assay (detailed in "Results"). Scientific graphing software (SigmaPlot version 1.02 for Windows, Jandel Scientific, San Rafael, CA) was used to fit the copper-uptake versus the copper-concentration data to the Michaelis-Menten equation, and to calculate kinetic parameters.

Cupric Reductase

The cupric reductase assay was developed from a previously described assay for yeast cupric reductase (Hassett and Kosman, 1995) with modifications to accommodate the growth conditions of *C. reinhardtii*. Cells from copper-deficient or copper-supplemented cultures in log-phase growth were collected by centrifugation (5000*g*, 3 min), washed, resuspended in copper-free TAP medium, and returned to the growth chamber (22°C, 250 rpm, approximately 150 μmol m^{-2} s^{-1}). Copper sulfate (chelated with an equimolar amount of EDTA) and BCDS were added from neutral stock solutions to final concentrations of 100 μM each. After 15 to 30 min, cells were collected by centrifugation and the amount of Cu(I) formed was determined by measuring the absorbance of the Cu(I)-BCDS complex at 480 nm (Blair and Diehl, 1961). Control experiments verified that product formation was dependent on the inclusion of Cu(II), BCDS, and cells in the assay. For the data shown in this work, the absorbance was normalized to the chlorophyll content in the assayed sample, and it is reported relative to the cupric reductase activity (A_{480} units min^{-1} mg^{-1} chlorophyll) in a copper-deficient culture assayed at the same time. The activity in copper-deficient cultures ranged from 0.06 to 0.12 A_{480} units min^{-1} mg^{-1} chlorophyll, which converts to a rate of 5 to 10 nmol Cu(I) min^{-1} mg^{-1} chlorophyll. A standard curve was used for the conversion. Each data point in the figure represents an average of 2 to 10 experiments. In each experiment all samples were assayed in duplicate, and the assay was linear for at least 60 min.

RESULTS

Copper Utilization by Copper-Deficient Cultures

C. reinhardtii cells have an extraordinary capacity to use even trace quantities of copper in the growth medium. In previous work we noted that the supplementation of copper-deficient medium with very low copper concentrations (1–10 nM) resulted in the use of that copper for holoplastocyanin synthesis (Merchant et al., 1991). The expression of the *CYC6* and *CPX1* genes was also affected, albeit transiently, in response to the added copper (Hill and Merchant, 1995). To assess their ability to use trace amounts of copper from the medium, copper-deficient *C. reinhardtii* cells were supplemented with copper (10–1200 nM) and sampled after supplementation to measure how much copper was left behind in the medium (Table I).

Remarkably, no residual copper, or only insignificant amounts, could be detected in the medium until the amount of supplied copper exceeded that required to saturate the plastocyanin biosynthetic pathway (approximately 9×10^6 copper ions/cell). For instance, in cultures containing approximately 4.6×10^6 cells/mL, approximately 69 nM copper is necessary for the synthesis of plastocyanin to its usual abundance, and until copper is supplied in excess of that demand, the cells appear to deplete the medium of all detectable copper. It is interesting that once that demand is met, copper uptake appears to be restricted so that it is not proportional to the supply (Table I, compare lines 6–8 in column A). This is compatible with the need to regulate copper uptake to prevent toxicity. As the cells divide to reach a density of approximately 8×10^6 cells/mL with a demand of about 120 nM copper for the plastocyanin biosynthetic pathway, copper uptake continues (Table I, lines 5 and 6, column A versus B) and is again restricted as the demand is met (Table I, compare lines 7 and 8 in column B). In some of the samples (e.g. lines 2–4 in column B) the residual copper in the medium may result from cell lysis as the culture reaches the stationary phase. The pattern of copper utilization from the medium suggested that uptake might be regulated in response to the supply of copper in the growth medium.

Table I. *Copper remaining in the growth medium of copper-supplemented cultures*

A single copper-deficient culture was split into eight aliquots, which were then supplemented with the indicated $CuSO_4$ concentrations from a standard stock of $CuSO_4$. The copper concentration in the medium was confirmed by the measurement of copper in a duplicate sample of uninoculated medium and was found to be within 5% of the indicated value. Duplicate analyses yielded nearly identical values (SD approximately 2–4% of the mean). The amount of copper in the copper-deficient culture (0) was subtracted from each sample to yield net copper. The amount of copper required to saturate the plastocyanin biosynthetic pathway at these cell densities is approximately 63 nM at 4.6×10^6 cells/mL and approximately 110 nM at 8×10^6 cells/mL. The amount of residual manganese, cobalt, and zinc (provided at the usual concentration in TAP medium) was also measured and was not significantly different between copper-supplemented samples versus copper-deficient samples.

Medium	Net Copper Remaining in Medium	
	4.6×10^6 cells/mL	8×10^6 cells/mL
nM	nM	
0	n.d.[a]	n.d.
10	n.d.	6.3
30	n.d.	15.7
60	n.d.	7.87
120	14	n.d.
240	24	4.7
571	294	41
1200	1000	554

[a] n.d., None detected; i.e. net copper content ≤ background of 1.6 nM.

Characterization of Copper Uptake

To directly characterize copper uptake by *C. reinhardtii* cells, a radioisotope of copper (^{64}Cu) was employed. Copper uptake was found to be temperature-dependent, and in copper-deficient cells, was linear with respect to time for at least 5 min at a substrate concentration of 20 nM (Fig. 1). In 10 min essentially all of the copper present in the assay medium is taken up, so further uptake cannot occur. For copper-replete cells, the assay was linear for at least 10 min at a substrate concentration of 20 nM (not shown), because the substrate is not depleted as rapidly. Upon a 100-fold dilution of the tracer with unlabeled $CuCl_2$, no efflux of radiolabeled copper was observed (Table II), which indicates that the copper is transported into the cell. If copper

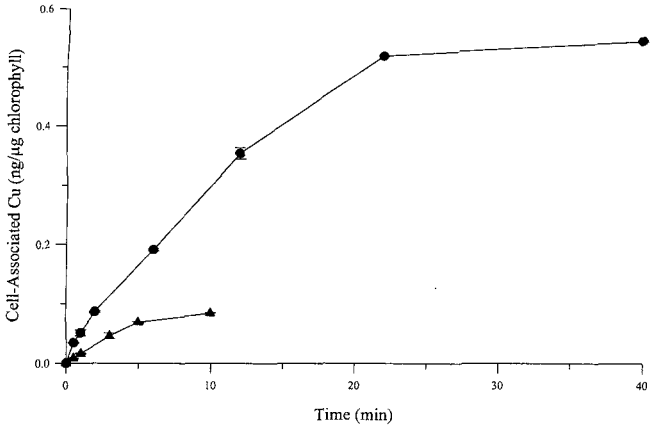

Figure 1. Time course of copper uptake in copper-deficient cells. Copper-deficient cultures were washed with uptake buffer (0.2 M Mes, pH 7.0) and resuspended in this buffer to approximately equivalent concentrations of chlorophyll. Copper-uptake assays were initiated at time 0 by the addition of $^{64}CuCl_2$ to a final concentration of 20 nM (▲) or 1 µM (●). Cell-associated copper was determined at the indicated time points as described in "Materials and Methods." Values reported are the means of duplicate samples. At the low substrate concentration (20 nM) samples were not collected beyond 10 min, because the substrate was entirely depleted by that time.

efflux is limited, the homeostatic control of internal copper levels is expected to occur by regulation of uptake. Indeed, at a high substrate concentration (1 µM), copper uptake by copper-deficient cells is essentially complete within 22 min, even though less than 10% of the copper present in the assay has been utilized (Fig. 1). The amount of copper taken up by this point in the assay corresponds to approximately 19×10^6 copper ions per cell, which is more than sufficient for the maintenance of the plastocyanin levels (Merchant et al., 1991). Therefore, cessation of uptake probably occurs because the demand for copper for utilization in copper-enzyme synthesis has been met.

Increased Copper Uptake Velocity in Copper-Deficient versus Copper-Replete Cells

To determine the concentration dependence of uptake, copper uptake was measured as a function of copper concentration in the assay (Fig. 2). Both copper-deficient and copper-sufficient cells exhibit a saturable copper uptake pathway, but the initial velocity of copper uptake is significantly increased in copper-deficient cells relative to copper-supplemented cells. Kinetic constants that were derived from a fit of these data to the Michaelis-Menten equation reveal that the increased rate of uptake by copper-deficient cells is not attributable to a higher affinity for copper (since the K_m for uptake is 210 ± 30 nM for copper-deficient cells versus 211 ± 48 nM for copper-supplemented cells), but rather to a higher capacity for uptake. In the experiment shown in Figure 2 copper-deficient cells exhibited about a 20-fold greater maximum velocity for copper uptake compared with copper-supplemented cells (62 ± 3 versus 2.9 ± 0.2 ng min^{-1} mg^{-1} chlorophyll). We conclude that *C. reinhardtii* cells possess an inducible, high-affinity copper uptake system, and that this system functions to provide copper only as needed for the maintenance of copper-protein levels.

Table II. *Cell-associated ^{64}Cu is not chased by the addition of excess, unlabeled $CuCl_2$*

Copper uptake was initiated by the addition of 100 nM $^{64}CuCl_2$ at time 0. Cell-associated copper was determined at the indicated time points as described in the legend to Figure 1. Immediately after removal of the sample at 3 min, unlabelled $CuCl_2$ was added to a concentration of 10 µM, and cell-associated copper was again determined at 4 and 8 min. Values reported are the averages of duplicate samples ± SD.

Time after Addition of ^{64}Cu	Time after Addition of Unlabeled Copper	Cell-Associated Copper
min	*min*	*ng/µg chlorophyll*
3	0	0.045 ± 0.005
4	1	0.037 ± 0.0001
8	5	0.039 ± 0.001

Increased Cupric Reductase Activity in Copper-Deficient Cells

The copper uptake machinery of other microorganisms includes, in addition to the copper-transport component, a cell-surface cupric reductase (Wakatsuki et al., 1988; Hasset

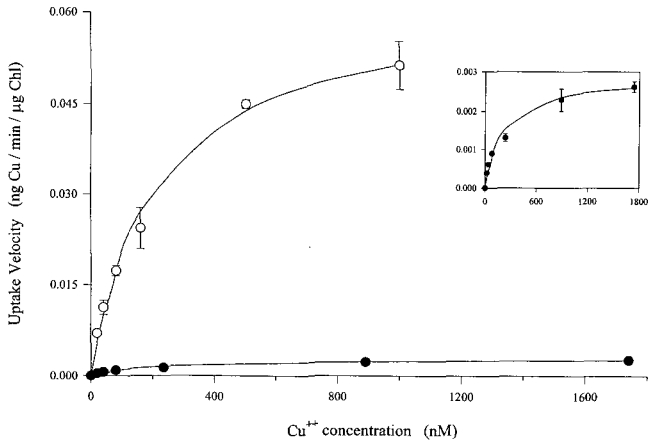

Figure 2. Concentration dependence of copper uptake in copper-deficient and copper-replete *C. reinhardtii* cells. Copper-deficient (○) or copper-replete (6 µM; ●) cells were washed and resuspended in uptake buffer and split into six aliquots each. Copper uptake was initiated by the addition of $^{64}CuCl_2$ to the final concentration indicated on the *x* axis (20 nM to 1.74 µM). After 1.17 and 3.17 min (copper-deficient samples) or 1 and 5 min (copper-replete samples), the amount of cell-associated copper was determined for each sample as described in "Materials and Methods," and the rate was determined from the difference. Values reported are the means of duplicate samples, and the lines represent the best fit of these data to the Michaelis-Menten equation. Inset, Expanded view of the copper uptake data obtained with copper-replete cells. The K_m was calculated to be 210 ± 30 nM for copper-deficient cells versus 211 ± 48 nM for copper-supplemented cells, whereas the maximum velocity was 62 ± 3 ng min^{-1} mg^{-1} chlorophyll (169 ± 9 pmol h^{-1} 10^6 $cells^{-1}$) versus 2.9 ± 0.2 ng min^{-1} mg^{-1} chlorophyll (8.16 ± 0.6 pmol h^{-1} 10^6 $cells^{-1}$).

Table III. *Cupric reductase activity is repressed by copper ion supplementation*

$[Cu^{2+}]$	Percent Activity[b] ± SE
μM[a]	
0	100 (10)[c]
0.05	71.5 ± 4.5 (2)
0.10	58.1 ± 5.5 (4)
0.20	52.3 ± 2.3 (2)
0.40	52.0 ± 1.0 (2)
3	51.3 ± 4.3 (2)
6	51.4 ± 2.2 (10)

[a] *C. reinhardtii* wild-type cells were sampled from copper-deficient medium (line 1) or from medium supplemented with the indicated $CuSO_4$ concentrations for 8 h. The average cell densities of the cultures at 0.05, 0.1, and 0.2 μM $CuSO_4$ were 8.5×10^6, 8.1×10^6, and 1.3×10^7 cells/mL, respectively. The corresponding ratios of copper ions/cell are thus 3.5×10^6 (0.05 μM), 7.4×10^6 (0.1 μM), and 9.3×10^6 (0.2 μM). [b] Cupric reductase activity was measured as described in "Materials and Methods." The activity is presented relative to that in copper-deficient samples, which were assayed in parallel for each experiment. [c] Numbers in parentheses indicate number of experiments.

and Kosman, 1995). This activity is easily assayed by a simple colorimetric method (Wakatsuki et al., 1991) that was applied to *C. reinhardtii* cells (Table III). Indeed, copper-deficient cells of *C. reinhardtii* exhibited increased reductase activity relative to cells that were maintained in the usual fully supplemented growth medium (6 μM). To assess whether the induction of cupric reductase activity might occur in coordination with other adaptations to copper deficiency, cells that were grown in medium supplemented with different copper concentrations were tested for cupric reductase activity. The amount of copper required to fully repress cupric reductase is in the same range that is required to fully repress copper uptake and *CYC6* and *CPX1* expression. In this case approximately 9×10^6 to 18×10^6 copper ions per cell (0.2–0.4 μM) suppressed the cupric reductase activity to the level observed in fully copper-supplemented cells (Table III).

Although the rate of copper transport is stimulated about 20-fold in copper-deficient cells, the rate of cupric ion reduction is increased only 2-fold. This might be expected if cupric reductase is not rate limiting for copper uptake. In fact, the 2-fold increase in the cupric reductase activity corresponds to an increase of 0.169 ng min^{-1} μg^{-1} chlorophyll in the rate of Cu(I) formation. This is almost 3-fold higher than the V_{max} of copper uptake by copper-deficient cells (0.062 ng min^{-1} μg^{-1} chlorophyll). Alternatively, it is possible that the "basal" rate observed with copper-supplemented cells is artificially high, because of a non-specific reduction by cell-surface reductants of the substrate, which is thermodynamically more disposed to reduction than free cupric ion (Hassett and Kosman, 1995).

The suppression of cupric reductase activity does not occur if copper is added to the medium immediately or shortly prior to assay; rather, it appears to require a few hours of adaptation to copper. This argues against the allosteric inhibition of cupric reductase by excess copper and suggests, instead, that the down-regulation of cupric reductase activity requires the dilution of the induced reductase. To test whether cupric reductase activity was coupled to an organellar electron transfer pathway, the assay was conducted in the presence of respiration or photosynthesis inhibitors. Antimycin A, an inhibitor of respiration, inhibited the inducible cupric reductase activity by only 20 to 30% (not shown), whereas DCMU, an inhibitor of photosynthesis, had no effect (Table IV). Cupric reductase activity in a photosynthesis-minus strain (FuD7) was, likewise, comparable to the activity in a wild-type strain (Table IV).

In the yeast *S. cerevisiae* copper and iron metabolism are closely linked, and the inducible metal reductases (e.g. Fre1p and Fre2p) that are required for metal assimilation can reduce both Cu(II) and Fe(III) (Eide et al., 1992; Askwith et al., 1994; Hasset and Kosman, 1995). Nevertheless, the cupric reductase and ferric reductase activities appear to be differentially responsive to copper and iron (Hassett and Kosman, 1995). To test whether this is the case in *C. reinhardtii*, the ferric reductase activity of copper-sufficient versus copper-deficient cells was compared. Copper supplementation inhibited the ferric reductase activity of iron-supplemented and iron-deficient cells to some extent (21 ± 6%) but not as much as it inhibited the cupric reductase activity of copper-deficient cells in the same experiments (51 ± 10%). Thus, the induction of cupric reductase activity in *C. reinhardtii* cannot be entirely attributed to the induction of iron-metabolizing enzymes in response to iron deficiency, and must, therefore, be a direct response to copper deficiency. This is expected, since copper-deficient *C. reinhardtii* cells show no evidence of iron deficiency. Iron-deficiency symptoms are distinct from copper-deficiency symptoms. For instance, the changes in protein synthesis profiles (not shown) or translatable mRNA profiles (Fig. 3) are unique for each type of trace element deficiency. The mRNA profiles of copper-deficient versus copper-supplemented cells (assessed by in vitro translation of total RNA) are not significantly different (Fig. 3, lane 2 versus lane 4). The only discernible change is an increase in the abundance of a translation product with an apparent molecular weight of 39×10^3 from copper-deficient RNA, corresponding probably to the precursor form of coprogen oxidase (Hill and Merchant, 1995). However, cells sub-

Table IV. *Cupric reductase activity is not directly coupled to photosynthesis*

Cells were maintained in copper-deficient medium (−Cu) or were supplemented with copper to a final concentration of 6 μM for 16 h (+Cu). Duplicate samples were removed from the culture for the measurement of cupric reductase activity.

Strain	Activity[a] ± SD	
	−Cu	+Cu
CC-124 (wild type)	5.4 ± 0.11 (3)[b]	2.69 ± 0.52 (3)
CC-124 + 3 μM DCMU	5.4 ± 0.46 (3)	2.18 ± 0.41 (3)
FuD7 (PSII-minus)	7.48	3.48

[a] Activity is reported as nmol Cu(I) min^{-1} mg^{-1} chlorophyll. [b] Numbers in parentheses indicate number of experiments.

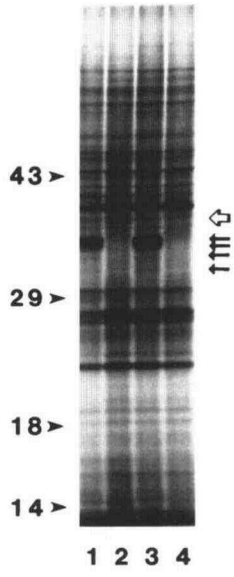

Figure 3. Induction of different mRNAs in iron-deficient versus copper-deficient cells. Total RNA (10 μg) was translated in vitro in a rabbit reticulocyte lysate system in the presence of [^{35}S]Met. The radiolabeled products of the translation reaction were analyzed by electrophoresis under denaturing conditions followed by fluorography for visualization, and the RNA was isolated from copper- or iron-deficient cells, as indicated. Copper deficiency was verified by measuring *CYC6* induction, and iron deficiency was verified by measuring the induction of ferric reductase activity. The arrowheads refer to the position of migration of standard marker proteins with molecular weights ($\times 10^{-3}$), as indicated. The thick open arrow points to an approximately 39-kD protein product, in which the abundance is increased in lanes 1 and 2 relative to lanes 3 and 4. The filled arrows point to protein products, in which the abundance is increased in lanes 1 and 3 relative to lanes 2 and 4.

jected to iron deficiency for only a few hours appear to accumulate several new mRNAs (Fig. 3, compare lane 3 with lane 4), and among these are the two that encode polypeptides with apparent molecular weights in the range of 33×10^3 to 35×10^3. Copper-deficient cells do not induce these iron-deficiency responsive RNAs. Thus, it is unlikely that copper deficiency in *C. reinhardtii* results in a secondary iron deficiency. The small amount of cross-inhibition of cupric and ferric reductases by copper and iron supplementation speaks, nevertheless, to an interrelationship between copper and iron metabolism in *C. reinhardtii*, which has already been established for other organisms (Jungmann et al., 1993; Robinson et al., 1993; Harris, 1994; Hassett and Kosman, 1995).

DISCUSSION

Copper Uptake

We have established the basic elements of a copper uptake pathway of *C. reinhardtii*. By measuring the uptake of a radioisotope of copper, we demonstrated that copper-deficient cells possess a saturable, temperature-dependent copper transport pathway that is suppressed in copper-sufficient cells. The affinity of the pathway for copper (K_m approximately 2×10^2 nM) is in the range expected, based on our previous studies of copper-responsive gene expression in this organism (Merchant et al., 1991), and is higher than that measured for *S. cerevisiae* with a similar assay (Lin and Kosman, 1990), although *S. cerevisiae* like *C. reinhardtii* has the ability to use copper from medium containing only nanomolar levels (Yuan et al., 1995).

Direct comparisons of the initial velocity of uptake in *C. reinhardtii* and *S. cerevisiae* are not possible from the work of Lin and Kosman (1990) (in which rates were reported in different units), but comparisons with the work of Dancis et al. (1994b) can be made. The rate of copper uptake, attributable to the yeast Ctr1p transporter, was estimated to be 8 pmol copper 10^6 cells^{-1} h^{-1}, which is about 20-fold slower than the rate calculated for *C. reinhardtii* in this work (Fig. 2). The greater affinity and the rate of copper uptake in *C. reinhardtii* may be necessary for scavenging copper ions from the natural growth environment, where it may be present at very low concentrations or where it may be necessary to compete with other organisms for the same resource. The wide distribution of species that respond to copper deficiency by using a Cyt c_6 in place of plastocyanin implies that copper deficiency is experienced in the natural habitat of these organisms (Sandmann et al., 1983). The magnitude and direction of the copper-responsive regulation of copper uptake is consistent with its involvement in an assimilatory pathway.

An interesting observation is that *C. reinhardtii* cells do not take up copper in great excess of what they need for the maintenance of copper protein levels, even when it is supplied in such excess. This is noted in experiments that are designed to assess copper uptake over the long term in a dividing culture (Table I) and also in experiments where the rate of copper uptake is measured directly (Fig. 1). In either case, copper uptake appears to be restricted once the cell has obtained an amount corresponding to between 1.9×10^7 and 4.9×10^7 copper ions per cell, which is about two to five times the amount needed to saturate the plastocyanin biosynthetic pathway. The regulation of copper transport may be a part of the mechanism for achieving copper homeostasis in this organism, as it is in bacteria (reviewed by Vulpe and Packman, 1995).

Cupric Reductase

In addition to copper transport, there appears to be a cupric reductase associated with *C. reinhardtii* cells. This activity is suggested to be a component of the copper-assimilation pathway because it is induced in copper-deficient cells. Although the role of Fe(III) reduction in the uptake of iron has been firmly established for yeast (Dancis et al., 1990; Anderson et al., 1992), and it is well accepted for plants and mammalian cells (e.g. Bruggemann et al., 1993; Guerinot and Yi, 1994; Randell et al., 1994), the involvement of cupric ion reduction is less certain but supported, nevertheless, by a number of experimental observations (Welch et al., 1993; Hassett and Kosman, 1995). For yeast, the reduction of periplasmic Cu(II) to Cu(I) by ascorbate suppresses the dependence of copper uptake on plasma membrane reductase activity. Suppression of the reductase activity also suppresses copper uptake. Copper

supplementation represses cupric reductase activity of yeast and plants, which is compatible with a role for the reductase in copper assimilation.

In this work we provide further support for the reductive assimilation of copper as a general feature of eukaryotic copper uptake pathways by demonstrating that cupric reductase activity is regulated in coordination with copper uptake, and more importantly, that the changes in copper transport and cupric reductase activity occur in response to physiologically relevant copper ion concentrations (Fig. 2; Tables I and III). Specifically, the same concentrations of copper are required to repress copper uptake and cupric reductase activity as are required for repression of other responses to copper deficiency, and the amount is related to the metabolic need of the cell (Hill and Merchant, 1992, 1995). We suggest that a common pathway might be involved in the regulation of *CYC6* and *CPX1* expression and copper uptake and cupric reductase activity. We hope to address this question in future work through the identification of copper sensors and copper-responsive regulators.

Although a cupric reductase activity has been measured in plants (e.g. Welch et al., 1993; Holden et al., 1995), it was not clear whether these activities are relevant to copper uptake. By studying copper uptake and cupric reductase in an organism with a well-characterized response to copper deficiency, it has been possible for us to correlate copper-responsive changes in cupric reductase activity with other changes that occur in response to copper supply, and to make a case for cupric reductase to be a general component of a copper-uptake pathway. It should be interesting to determine whether the cupric reductase of plants is regulated in coordination with putative copper transporters (see Kampfenkel et al., 1995).

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