Copper economy in *Chlamydomonas*: Prioritized allocation and reallocation of copper to respiration vs. photosynthesis

Janette Kropat, Sean D. Gallaher, Eugen I. Urzica, Stacie S. Nakamoto, Daniela Strenkert, Stephen Tottey, Andrew Z. Mason, and Sabeeha S. Merchant

*Department of Chemistry and Biochemistry, and Institute for Genomics and Proteomics, University of California, Los Angeles, CA 90095; and Institute for Integrated Research in Materials, Environments, and Societies, California State University, Long Beach, CA 90840*

This contribution is part of the special series of Inaugural Articles by members of the National Academy of Sciences elected in 2012.

Contributed by Sabeeha S. Merchant, January 13, 2015 (sent for review October 24, 2014; reviewed by Jonathan Gitlin, Jean-David Rochaix, and Rosemary Stuart)

Inorganic elements, although required only in trace amounts, permit life and primary productivity because of their functions in catalysis. Every organism has a minimal requirement of each metal based on the intracellular abundance of proteins that use inorganic cofactors, but elemental sparing mechanisms can reduce this quota. A well-studied copper-sparing mechanism that operates in microalgae faced with copper deficiency is the replacement of the abundant copper protein plastocyanin with a heme-containing substitute, cytochrome (Cyt) C₆. This switch, which is dependent on a copper-sensing transcription factor, copper response regulator 1 (CRR1), dramatically reduces the copper quota. We show here that in a situation of marginal copper availability, copper is preferentially allocated from plastocyanin, whose function is dispensable, to other metal-copper-dependent enzymes like Cyt oxidase and a ferroxidase. In the absence of an extracellular source, copper allocation to Cyt oxidase includes CRR1-dependent proteolysis of plastocyanin and quantitative recycling of the copper cofactor from plastocyanin to Cyt oxidase. Transcriptome profiling identifies a gene encoding a Zn-metalloprotease, as a candidate effecting copper recycling. One reason for the retention of genes encoding both plastocyanin and Cyt C₆ in algal and cyanobacterial genomes might be because plastocyanin provides a competitive advantage in copper-depleted environments as a ready source of copper.

Copper homeostasis | acclimation | RNA-seq | metal | copper store

Many inorganic elements, because they are required for catalysis or as structural components in biomolecules, are essential nutrients for life. In a situation of poor nutrition, organisms economize on the use of these elements by choosing alternate chemistries to achieve the same function. The mechanism of copper economy in *Chlamydomonas* involves replacement of copper-containing plastocyanin with heme-containing cytochrome (Cyt) C₆. The copper that is saved by this substitution is used instead for Cyt oxidase biosynthesis. Copper recycling requires proteolysis of plastocyanin dependent on a copper-sensing transcription factor. A candidate protease has been identified.

Significance

Many inorganic elements, because they are required for catalysis or as structural components in biomolecules, are essential nutrients for life. In a situation of poor nutrition, organisms economize on the use of these elements by choosing alternate chemistries to achieve the same function. The mechanism of copper economy in *Chlamydomonas* involves replacement of copper-containing plastocyanin with heme-containing cytochrome (Cyt) C₆. The copper that is saved by this substitution is used instead for Cyt oxidase biosynthesis. Copper recycling requires proteolysis of plastocyanin dependent on a copper-sensing transcription factor. A candidate protease has been identified.


Reviewers: J.G., Marine Biology Laboratory; J.-D.R., University of Geneva; and R.S., Marquette University.

The authors declare no conflict of interest.

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1Present address: Institute of Plant Biology and Biotechnology, Westfälische Wilhelms University, 48143 Muenster, Germany.

2Present address: Fraunhofer USA Center for Molecular Biotechnology, Newark, DE 19711.

3To whom correspondence should be addressed. Email: sabeeha@chem.ucla.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1422492112/-/DCSupplemental.
protein constitutes a large fraction of the copper quota (17, 18). In a copper-deficient situation, plastocyanin function can be replaced by cytochrome (Cyt) C6, a functionally equivalent heme (iron)-containing e-type Cyt. The replacement makes the copper protein nonessential for photosynthesis and reduces the copper quota (15).

The copper-acquiring pathway has been well-elucidated in the reference organism *Chlamydomonas* (20). The PCY1 gene encoding plastocyanin is constitutively expressed, but the protein accumulates only in copper-replete cells; in copper-deficient cells, the protein is rapidly degraded (19, 21). The CYC6 gene encoding Cyt C6 is transcribed exclusively in copper-deficient cells to cover the loss of plastocyanin function (22). CYC6 expression occurs via transcriptional activation dependent on copper-response elements and a copper-sensing transcription factor, copper response regulator 1 (CRR1), which binds to such elements associated with the CYC6 promoter (23, 24).

A survey of the *Chlamydomonas* genome revealed dozens of candidate cuproproteins, but transcriptional and proteome analyses indicate that the copper quota of the cell is dependent largely on a few (25). PCY1 mRNA is one of the most abundant transcripts in the cell (top 10 of >17,000 genes), consistent with the abundance of plastocyanin (~8 × 10^6 molecules per cell). Other abundant transcripts include FOXI, encoding a ferroxidase required for iron assimilation, and hence more heavily expressed in iron-deficient cells; COX2A and COX2B, encoding one of two copper-binding subunits of Cyt oxidase, which is essential for respiration in the mitochondria; and UOX1 encoding urate oxidase, which functions in nitrogen metabolism. Quantitative proteomics of soluble fractions confirmed the dominance of plastocyanin over other cuproproteins (e.g., 100-fold higher than urate oxidase), which underscores its dominant contribution to the total cellular copper quota (26).

In this study, we manipulate the cellular copper quota by growing cells under conditions where one of three major copper-using pathways is prioritized (photosynthesis with plastocyanin, iron assimilation with ferroxidase, and respiration with Cyt oxidase) to probe the dynamics of copper distribution to these pathways. Copper is preferentially allocated to Cyt oxidase (whose proton-pumping function cannot be replaced by a copper-independent enzyme) over plastocyanin and, in the absence of a nutritional supply, can be recycled from plastocyanin in a CRR1-dependent pathway.

**Materials and Methods**

**Strains and Culture Conditions.** *Chlamydomonas reinhardtii* WT strain CC-4532, WT strain CC-124, *dum19* mutant strain CC-3400, *cri2-1* mutant strain CC-3960, and the complemented *cri2-1* (CRR1) strain CC-5071 were cultured in copper-deficient (20 nM) or copper-supplemented (400 nM) tris-acetate-phosphate (TAP) medium with Hutner’s trace elements as described previously (27). Cultures were incubated at 24 °C with 180 rpm agitation and 120 μmol of photons per m^-2^ s^-1^ of continuous illumination under 4,100-K cool-white fluorescent bulbs. Where indicated, copper-deficient medium was supplemented to the desired amount with EDTA-chelated CuCl₂. For iron-deficient cultures, cells were precultured to stationary phase in deficient medium (1 μM Fe) (28). For photoautotrophic growth, acetate was eliminated from TAP medium and the pH was adjusted to 7.4 with HCl. For heterotrophic growth, flasks were wrapped in aluminum foil.

**Subfractionation of Cells.** *Chlamydomonas* cultures were collected by centrifugation (1,000 × g for 5 min) and washed twice with ultrapure 4 mM sodium phosphate, pH 7.0. Quantitative fractionation of cells into soluble and membrane components is described by Howe and Merchant (29). After the first separation, the pellet was washed with an equal volume of phosphate buffer and resuspended by centrifugation. The supernatants were combined and further clarified three times by centrifugation. The pellet fractions at each step were combined. Immunoblot analyses confirmed that the membrane fraction was free of plastocyanin or Cyt C6 and the soluble fraction was free of Cyt oxidase. For samples that were subsequently analyzed for metal content, the cells were washed with 1 mM EDTA before the washes with phosphate buffer and fractionation. All tubes used in these experiments were washed with soap, rinsed with deionized water (Biocel A10; Millipore), and soaked for 7 d in 10% (wt/vol) nicotinic acid, followed by a second rinsing with deionized water.

**Preparation of COX2B Antiserum.** A 447-bp cDNA fragment corresponding to amino acid residues 4–153 of COX2B (GenBank accession no. AF305543) was amplified using the primers 5’-gctagctaggctcAGGACCACTGAAAGAGAAG-3’ and 5’-gtagcttccagtGACCATTCTCCTGACGTA-3’ in a 50-μL PCR assay consisting of 1 μL of plasmid DNA, 32 pmol of each primer, 10 nmol of dNTPs, and 2.5 units of Taq polymerase. The amplification conditions were 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1.5 min. The amplified fragment was cloned into the BamHI and EcoRI sites of the pGEX4T-1 vector (GE Healthcare) and introduced into Escherichia coli for isopropyl-p-θigalactopyranoside-inducible expression. The expressed GST-fusion protein was found in inclusion bodies and was purified by preparative SDS gel electrophoresis. Rabbits were immunized via s.c. nodal injections (200 μg of purified fusion protein with Freund’s complete adjuvant). Antiserum specificity was demonstrated by immunoblotting of protein extracts from *C. reinhardtii* WT CC-124 and the *dum19* mutant (strain CC-239, which lacks Cyt oxidase) and by signal competition with antigen. All antibodies used in this work are available at Agrisera AB.

**Immunoblot Analysis.** For immunoblot analysis, proteins were separated on SDS-containing polyacrylamide gel (7.5% monomer for FOXI; 10% monomer for Cyt C6, and 15% monomer for plastocyanin, Cyt COX2B, and COX2B) and transferred to nitrocellulose membrane (1 μm; Whatman GmbH) using a semidyey blottter under constant power (4 W) for 30 min (plastocyanin, Cyt C6, and COX2B), 1 h (Cyt), or 1.5 h (FOXI) in 25 mM Tris, 192 mM Gly, and 2% (v/v) methyl methanol with 0.1% SDS. For Cyt C6 or without 0.01% SDS (plastocyanin, Cyt COX2B, and COX2B). All membranes were blocked overnight with 1% calf serum (HyClone) in PBS (9.5 mM phosphate, 8 mM sodium, 4.5 mM potassium, and 140 mM NaCl). Primary antibody concentrations were 1:300 (FOXI), 1:500 (plastocyanin and Cyt C6), 1:5,000 (COX2B), and 1:10,000 (Cyt C6) dilution in 1% nonfat milk in PBS with 0.05% (v/v) Tween 20 (wt/vol). A secondary antibody, goat anti-rabbit alkaline phosphatase (1:3,000 dilution; Southern Biotech), was used for colorimetric detection with NBT and BCIP. Unless stated otherwise, protein extracts from a fixed number of cells were analyzed (5 × 10^6 cells for Cyt C6 and FOXI, 3 × 10^7 cells for plastocyanin, 2 × 10^7 cells for COX2B, and 3 × 10^5 cells for Cyt C6). Loading based on equal cell numbers is valid because the cell size distribution in the cultures, although broad, did not change in the various conditions used. Those amounts were chosen for a linear range for detection of each protein. To permit a fair comparison between samples within an experiment, all individual gels were transferred onto a single membrane and processed simultaneously.

**Metal Measurement.** *Chlamydomonas* cells grown to the indicated cell density or to stationary phase (>1 × 10^7 cells per milliliter) were collected and washed once with phosphate buffer; after centrifugation, the supernatant and pellet fractions were combined with the initial soluble or insoluble fractions. For measurements of the metal content of the spent medium, cells were collected in acid-washed tubes and part of the remaining medium was carefully transferred into fresh acid-washed tubes. Residual cells were removed by centrifugation (two times sequentially at 20,000 × g for 10 min) before the addition of nitric acid. The metal content was analyzed using a PE-6100DRC ICP-MS system (PerkinElmer) (SI Text).

**RNA-sequencing Analysis.** RNA-sequencing (RNA-seq) libraries were prepared from total RNA and sequenced in 35-nt reads at Illumina. The resulting sequence data were filtered to remove the 3’ adaptor sequence with scythe ([https://github.com/vsbuffalo/scythe](https://github.com/vsbuffalo/scythe)) and low-quality nucleotide calls (<15) with sickle ([https://github.com/vsbuffalo/sickle](https://github.com/vsbuffalo/sickle)). Next, reads were aligned to version 5 of the *C. reinhardtii* reference genome ([http://phytozome.jgi.doe.gov/](http://phytozome.jgi.doe.gov/)) using Tophat2 (31). The quality of the alignment was evaluated by visualization with the Integrative Genomics Viewer browser (32). Gene expression estimates were calculated in terms of fragments per kilobase of transcript
per million mapped reads (FPKMs) by cuffdiff (33). Genes whose expression was 10 FPKMs or greater and whose expression increased eightfold or more, or were coexpressed with CYC6, were identified with the cummerBund package of Bioconductor and plotted as a clustered heat map using the R programming language (34).

**Results**

We can demonstrably manipulate the copper quota of *Chlamydomonas* by changing physiological demand for one of three abundant copperproteins (Fig. 1). The biogenesis of the photosynthetic apparatus does not require light, but it is promoted by illumination coincident with a two- to threefold increased capacity for photosynthesis (Table 1). The capacity for oxygen consumption, on the other hand, is increased (about twofold) when cells are provided with acetate, whose utilization requires respiration. The abundance of the copper proteins plastocyanin and Cyt oxidase in these bioenergetic pathways parallels the change in pathway capacity (Fig. 2) and contributes to the variation in cellular copper quota. The quota is further increased in iron-poor medium (Fig. 1) when the expression of a high-affinity pathway consisting of a copper-requiring ferroxidase coupled to a ferric transporter is induced (35).

Each of these copperproteins is located in a different compartment in the cell and uses distinct pathways for copper delivery. The ferroxidase (encoded by *FOX1*) is anchored to the plasma membrane and has an extracellular active site with six copper atoms, which are loaded via the copper chaperone ATX1. A ferric transporter is induced (35).

When copper is supplied below the quota, the driving force for in vivo copper protein synthesis (39) results in complete depletion from the medium (Table 2). For instance, 20 nM copper is inadequate to support plastocyanin accumulation at a density of $6 \times 10^6$ cells per milliliter, yet the abundance of Cyt oxidase is maintained as the culture divides to reach a density of $12 \times 10^6$ cells per milliliter (Fig. 3). Maintenance of Cyt oxidase occurs coincident with the complete loss of plastocyanin, implicating plastocyanin as the source of copper for Cyt oxidase synthesis (<3%; Fig. S3).

To demonstrate the importance of plastocyanin as the source of copper for de novo Cyt oxidase synthesis, we quantitatively monitored copper during growth of a batch culture in a situation of limiting copper nutrition, analogous to an algal bloom in nature. We chose 20 nM copper because it was sufficient for plastocyanin accumulation at early log phase but inadequate at late log phase (Fig. 3). The medium contained ammonium (which minimizes urate oxidase expression) and was supplemented with a ChloraLab2 electrode from Hansatech. The rate of oxygen production was measured after the addition of 10 mM potassium hydrogen carbonate and illumination with 300 μmol of photons per m$^{-2} \cdot$s$^{-1}$ in 2 mL of culture. After measurement of activities, the cells were collected and the copper content was determined by inductively coupled plasma (ICP)-MS. The values represent the mean and SD of three independent experiments.

**Table 1. Light and carbon source affect capacity for photosynthesis and respiration**

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Respiratory rate, nmol of O$_2$ per minute per 10$^6$ cells</th>
<th>Photosynthetic rate, nmol of O$_2$ per minute per 10$^6$ cells</th>
<th>Cu content, ×10$^7$ atoms per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light acetate</td>
<td>35.6 ± 7.0</td>
<td>42.9 ± 6.7</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>Light CO$_2$</td>
<td>19.0 ± 1.3</td>
<td>33.1 ± 4.0</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>Dark acetate</td>
<td>34.7 ± 3.2</td>
<td>12.0 ± 1.8</td>
<td>2.3 ± 0.1</td>
</tr>
</tbody>
</table>

For phototrophic growth, WT strain CC-4532 was grown in acetate containing medium in the light to a density of $3 \times 10^6$ cells per milliliter. For heterotrophic or phototrophic growth, the strain was grown in the dark or without acetate, respectively. Two milliliters of culture was removed; after addition of 34 mM acetate, oxygen consumption in the dark was analyzed with a ChloraLab2 electrode from Hansatech. The rate of oxygen production was measured after the addition of 10 mM potassium hydrogen carbonate and illumination with 300 μmol of photons per m$^{-2} \cdot$s$^{-1}$ in 2 mL of culture. After measurement of activities, the cells were collected and the copper content was determined by inductively coupled plasma (ICP)-MS. The values represent the mean and SD of three independent experiments.
with excess iron (200 μM) to repress ferroxidase accumulation; thus, in these conditions, plastocyanin and Cyt oxidase represent the bulk of the cellular cuproprotein content. We measured the copper content of the spent medium vs. intracellular copper separated into the supernatant fraction containing plastocyanin and the membrane fraction containing Cyt oxidase. Freeze–thaw rupture of cells followed by centrifugation allowed quantitative separation and recovery of plastocyanin into the supernatant fraction and Cyt oxidase into the pellet fraction, which were also probed by immunoblot analyses for plastocyanin, Cyt c6, and COX2B (Fig. 4). In parallel, the culture was also sampled to assess its capacity for photosynthesis (O₂ evolution) and respiration (O₂ consumption) (Table 3).

At an early stage in the bloom, when the cells are in a copper-replete state, the copper contents of plastocyanin and Cyt oxidase increase with increasing cell density (0.5–1 × 10⁶ cells per milliliter), whereas the spent medium shows a corresponding decrease in copper. At 2 × 10⁶ cells per milliliter, the medium is completely depleted of copper. However, Cyt oxidase content and the copper content of the membrane fractions continue to increase as the cells grow and divide. The increase occurs at the expense of plastocyanin (Fig. 4E) and the copper content of the supernatant fraction (Fig. 4B). Quantitative mass recovery of the copper content of the partitioned samples (Fig. 4D) is consistent with plastocyanin as the only source of copper for de novo Cyt oxidase synthesis. This scavenging mechanism allows the cells to maintain the capacity for respiration (Table 3). The loss of plastocyanin does not compromise photosynthetic capacity, even at the switch point, because Cyt c6 accumulation is concomitant with plastocyanin loss (4 × 10⁶ cells per milliliter) (Fig. 4E and Table 3). Because the immunoblot assay for plastocyanin is linear over a 30-fold concentration range (Fig. S3), the disappearance of plastocyanin in a single round of cell division (Fig. S3 vs. Fig. 3 and Fig. 4E) is consistent with active degradation of the protein (40).

To reveal the factors in copper mobilization during an algal bloom in a copper-limited situation, we profiled the *Chlamydomonas* transcriptome by RNA-seq during the transition from copper-replete (0.5 × 10⁶ and 1 × 10⁶ cells per milliliter) to copper-deficient (4 × 10⁶ and 8 × 10⁶ cells per milliliter) conditions (as in the experiment shown in Fig. 4) in cultures supplemented with 20 nM copper. To control for changes in gene expression that are dependent on cell density, we monitored, in parallel, cultures supplemented with 400 nM copper, which is saturating for the synthesis of all copper proteins (Fig. 3 and Fig. S2). Sequenced reads were aligned to the *Chlamydomonas* genome and used to estimate gene expression in terms of FPKMs. In previous work based on a two-way comparison of WT and *crr1* cells acclimated to copper deficiency, we had identified 63 genes that were likely targets of *CRR1* (25). Only 56 of these genes were mapped forward to the latest version of the *Chlamydomonas* genome, and 90% of these genes (50 of 56) show increased expression as cells divide in 20 nM copper but not as they divide in 400 nM copper (Fig. 5). Among these genes are *CYC6* and *CPX1*, which are sentinel genes of the copper nutrition signaling pathway and, not surprisingly, a number of candidate metal transporters. The *CTRs*, which are involved in copper assimilation (25-fold increase for *CTR2*, eightfold increase for *CTR3*, and fourfold increase for *CTR1*), and ZIP family transporters (23-fold increase for *IRT2*, fourfold increase for *ZRT2*, and threefold increase for *ZIP7*) are especially notable. Previously, we had suggested that *IRT2* (whose expression is also regulated by iron deficiency) may serve as a back-up of the FOX1/FTR1 pathway (41). The *CTRs* do show slightly increased expression in copper-replete cells, presumably because cell division depletes the medium of copper, but the induction is less pronounced than the induction observed under copper deficiency. Although copper export from the chloroplast should be catalyzed by a transporter, we were unable to identify a candidate gene for this function from the transcriptome analysis. It is certainly possible that export occurs by passive transport relying on a constitutively expressed transporter. Only one gene, *CTH1* (encoding an enzyme in chlorophyll biosynthesis), appeared to be down-regulated in copper deficiency. *CTH1* is also a target of *CRR1*; its expression is reciprocal to the expression of its homolog, *CRD1*, whose expression increases in copper deficiency (42).

When we expand this analysis to include all expressed genes (≥10 FPKMs) that increased eightfold or more over the course of the experiment, we identified an additional 12 genes that increased in expression as copper becomes depleted in the 20 nM cultures. Two of these genes, *HYDEF* (25-fold increase) and *AOX2* (26-fold increase), were identified previously as being regulated by copper but were not known to be *CRR1*-dependent because these genes were also regulated in *crn1* (25). The remaining 10 copper-regulated genes [Cre01.g004750, Cre01.g029700, Cre03.g203569, Cre05.g241647, Cre05.g244000, Cre07.g350976, Cre10.g426800, *CBD1* (listed only as Cre14.g609900 in Phytozome version 10), Cre16.g656750, and Cre17.g734644] were not identified in that earlier study. In support of the hypothesis that these genes are regulated by *CRR1*, all had one or more putative copper response elements (CuREs) in the 300 nt upstream of the transcription start site (25).

We were particularly interested in identifying the protease that degrades plastocyanin to liberate its copper. Copper is very tightly

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**Fig. 2.** Plastocyanin and Cyt oxidase abundance per cell is dependent on light and acetate. For photoheterotrophic growth, WT strain CC-4532 was grown in acetate-containing medium with 6 μM copper in the light to a density of 6 × 10⁶ cells per milliliter. For heterotrophic or phototrophic growth conditions, the strain was grown in the dark or without acetate, respectively. Cells were collected by centrifugation, and plastocyanin and COX2B abundances in an equal number of cells (3 × 10⁶ cells and 2 × 10⁶ cells, respectively) were analyzed by immunoblotting. A representative blot of a dilution series from an experimental triplicate is shown.

**Fig. 3.** Hierarchical accumulation of copper-containing proteins in copper-deficient cells. Cells of strain CC-4532 were grown in acetate-containing medium supplemented with the indicated concentrations of copper and 1 μM iron, Cells from the same culture were collected at the indicated densities, and the abundances of plastocyanin (PC), Fox1, COX2B, and the ωj subunit of CFT (ωj/CFTj) in 1 × 10⁶ cells (per lane) were monitored by immunoblotting.
bound to plastocyanin and does not dissociate in a physiologically relevant time scale; furthermore, previous work invoked a process involving regulated degradation (40). Given the tight reciprocal accumulation of plastocyanin vs. Cyt c₆ (Fig. 4E), we expect the degradative activity to be expressed in parallel with the expression of CYC6. Therefore, we searched the dataset for genes coregulated with CYC6 (Fig. S5). The most likely candidate, RSEP1, named for the relationship of the encoded protein with a bacterial membrane-localized zinc metalloprotease called RseP (43), clusters closely with CYC6. Its expression increases 54-fold as copper is depleted in the 20 nM cultures. RSEP1 belongs to the S2P family of membrane-bound metalloproteases, which includes At2g32480 in Arabidopsis that is localized to the inner chloroplast envelope membrane and required for chloroplast development (44). Orthologs of Arabidopsis At2g32480 are found in all plants, including Chlamydomonas (RSEP2) and other green algae. RSEP1 distribution, on the other hand, is restricted to Chlamydomonas and Volvox, consistent with a specialized function, perhaps in plastocyanin degradation. The protein is predicted to be targeted to an organelle: chloroplast according to TargetP and S2P protease subcellular localization according to Predalgo. Expression of RSEP1 in Chlamydomonas is directly responsive to copper nutrition through CRR1, and immunoblot analysis confirms that the protein accumulates in parallel with the RNA. Nevertheless, the protein appears to be unstable, which has precluded fractionation and purification. Conclusive demonstration of function awaits reverse genetic analysis; to date, knock-down experiments have not substantially reduced RSEP1 abundance. The founding member of this family of proteases is the E. coli RseP, which targets transmembrane domains of proteins subsequent to their cleavage by a S1P protease (DegS) (45). There are several Deg proteases in the chloroplast, including in the thylakoid lumen, and it is possible that RSEP1 works in concert with one of these Deg proteases (46). An active site near the membrane surface would allow it to act on a soluble protein like plastocyanin, which is known to interact with the membrane (43).

We took advantage of the crr1 mutant to validate the role of the copper regulon in maintenance of the copper proteome of Chlamydomonas. The mutant is completely deficient in the activity of CRR1, which is a transcription factor that recognizes CuREs and is required for activating the copper regulon in copper deficiency (23). Although the mutant grows poorly in copper deficiency (Fig. S7), it is possible to collect sufficient material for biochemical analysis of protein abundance. The samples were analyzed by nondenaturing PAGE to separate holo-plastocyanin from apo-plastocyanin as well as denaturing gels to monitor COX2B (Fig. 6). The crr1 mutants are unable to maintain Cyt oxidase levels at a low nutritional supply of copper relative to the complemented strain. Indeed, even with 400 nM copper in the medium, plastocyanin expression is directly responsive to copper nutrition through CRR1, and immunoblot analysis confirms that the protein accumulates in parallel with the RNA. Nevertheless, the protein appears to be unstable, which has precluded fractionation and purification. Conclusive demonstration of function awaits reverse genetic analysis; to date, knock-down experiments have not substantially reduced RSEP1 abundance. The founding member of this family of proteases is the E. coli RseP, which targets transmembrane domains of proteins subsequent to their cleavage by a S1P protease (DegS) (45). There are several Deg proteases in the chloroplast, including in the thylakoid lumen, and it is possible that RSEP1 works in concert with one of these Deg proteases (46). An active site near the membrane surface would allow it to act on a soluble protein like plastocyanin, which is known to interact with the membrane (43).

![Figure 4. Reallocation of copper from plastocyanin to Cyt oxidase. Copper-deficient cells of strain CC-4532 were inoculated into TAP medium containing 20 nM supplemental copper to a density of 1 × 10⁶ cells per milliliter. The culture was sampled (volume equivalent to 2 × 10⁶ cells) at 0.5 × 10⁶, 1 × 10⁶, 2 × 10⁶, 4 × 10⁶, 8 × 10⁶, and 16 × 10⁶ cells per milliliter. (A) Total copper content of 1 L of spent medium after removal of cells (the lane marked with medium refers to the medium before inoculation). Supernatant (containing plastocyanin) and pellet (containing Cyt oxidase) fractions of the cells were quantitatively recovered and analyzed for copper content (B and C) and abundance of plastocyanin, Cyt c₆, and Cyt oxidase (E). For B and C, the y axis shows the copper content of the supernatant and pellet fractions of cells in 1 L of culture. (D) Sum of the copper content from A–C. The experiment was conducted in biological triplicate, and the values show the average ± SD. For E, the sample was loaded on the basis of an equivalent volume of culture and only one representative blot is shown. The soluble fraction was also analyzed by HPLC, both by size-exclusion and anion-exchange HPLC (copper repletion is shown in Fig. S4) coupled to the ICP-MS. All copper-containing peaks could be attributed to either free copper or plastocyanin in various samples (copper-replete and copper-deficient soluble extract from a mutant lacking plastocyanin, purified plastocyanin, and an aliquot of copper solution used to supplement the medium).](image-url)
copper supplementation, which is adequate in the WT (discussed above), 
crr1 is unable to accumulate its full complement of cuproproteins. The loss of Cyt oxidase likely results both from reduced copper assimilation (because of an inability to activate the CTR genes) and the absence of a holo-plastocyanin pool from which to harvest copper (Fig. 6). The plastocyanin polypeptide Table 3. Photosynthesis and respiration capacity is maintained during a Cu-limited algal bloom

<table>
<thead>
<tr>
<th>Cell density, ×10^6 cells per milliliter</th>
<th>Respiration</th>
<th>Photosynthesis</th>
<th>Respiration</th>
<th>Photosynthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol of O₂ min⁻¹·mL⁻¹</td>
<td>nmol of O₂ per minute per 10⁷ cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>1.0 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>16.6 ± 1.7</td>
<td>22.2 ± 1.9</td>
</tr>
<tr>
<td>1</td>
<td>1.4 ± 0.2</td>
<td>3.4 ± 0.4</td>
<td>13.8 ± 1.9</td>
<td>34.0 ± 5.1</td>
</tr>
<tr>
<td>2</td>
<td>2.6 ± 0.3</td>
<td>6.7 ± 2.0</td>
<td>13.4 ± 1.2</td>
<td>34.7 ± 9.7</td>
</tr>
<tr>
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<td>12.9 ± 0.5</td>
<td>14.1 ± 3.2</td>
<td>32.0 ± 0.9</td>
</tr>
<tr>
<td>8</td>
<td>12.1 ± 3.0</td>
<td>29.5 ± 4.5</td>
<td>16.2 ± 1.7</td>
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<tr>
<td>16</td>
<td>22.1 ± 1.2</td>
<td>47.9 ± 1.7</td>
<td>18.7 ± 1.5</td>
<td>36.8 ± 3.8</td>
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</tbody>
</table>

Copper-deficient cells of strain CC-4532 were inoculated into TAP medium containing 20 nM supplemental copper to a density of 1 × 10⁶ cells per milliliter. When cells reached 0.5 × 10⁶ cells per milliliter, and subsequently at 1 × 10⁶, 2 × 10⁶, 4 × 10⁶, 8 × 10⁶, and 16 × 10⁶ cells per milliliter (corresponding to one doubling each time), 2 mL of culture was removed and analyzed with a ChloroLab2 electrode from Hansatech. Oxygen consumption in the dark was measured for a period of 10 min after the addition of 17 mM acetate. After the addition of 10 mM potassium bicarbonate, the cultures were illuminated with 300 μmol of photons per m⁻²·s⁻¹ and the rate of oxygen evolution was observed for an additional 10 min. The values shown represent the average and SD of three independent experiments.

Fig. 5. CRR1 targets are differentially expressed during the transition from copper repletion to copper depletion. WT strain CC-4532 was inoculated at 1 × 10⁶ cells per milliliter into TAP medium supplemented with either 20 or 400 nM copper. RNA was purified from cells of each culture once the cell density reached 0.5 × 10⁶, 1 × 10⁶, 2 × 10⁶, 4 × 10⁶, and 8 × 10⁶ cells per milliliter. RNA was quantified in terms of FPKMs by RNA-seq. Expression estimates from 56 genes that had previously been identified as targets of the CRR1 transcription factor are plotted as a clustered heat map. Log₁₀ transformations of FPKMs are presented on a scale ranging from blue (lowest expression) to yellow (highest expression). AOF1, amine oxidase, flavin-containing 1; CGL7B, conserved in green lineage 78; CPX1, coproporphyrinogen III oxidase 1; CRD1, copper response defect 1; CTH1, copper target homolog 1; CTR1, copper transporter 1; FDX5, ferredoxin 5; GOX3, glyoxal or galactose oxidase 3; HPD1, 4-hydroxyphenylpyruvate dioxygenase 1; IRT2, iron responsive transporter 2; VPS35, vacuolar protein sorting-associated protein 35; XUV6, xanthine/uracil/vitamin C permease-like protein 6; ZIP7, zinc/copper transporter 7; ZRT2, zinc responsive transporter 2.
accumulates to substantial levels, although largely in the nonfunctional apoform (presumably because of reduced copper assimilation capacity of the crr1 strain). Any holoprotein that is made appears not to be degraded in copper-deficient cells of crr1. Accordingly, copper cannot be salvaged for maintenance of Cyt oxidase. Thus, crr1 mutants fail to grow either phototrophically or heterotrophically in copper-poor medium (Fig. S7), which reinforces the importance of CRR1 in nutritional copper homeostasis in the plant lineage. Note that RSEP1 is not induced in crr1, which is not inconsistent with the hypothesis that RSEP1 might encode a plastocyanin-degrading protease (25).

Discussion

Metabolism Defines the Copper Quota. Three cuproproteins, plastocyanin, ferroxidase, and Cyt oxidase, each with a function in fundamental metabolic pathways (photosynthesis, iron assimilation, and respiration, respectively) are largely responsible for the copper quota in Chlamydomonas. Their contribution is evident from the change in copper quota in response to metabolic changes that affect the expression of the three cuproproteins. When acetate is provided in the medium, which allows respiratory growth, mitochondrial biogenesis is stimulated and Cyt oxidase stoichiometry and the associated copper content per cell increase two- to threefold, with an increase in the copper quota of the cell (Figs. 1 and 2 and Table 1). Although these experiments do not identify the cellular location of the copper, measurements of respiratory activity confirm an increase, indicating that at least some of the additional copper must be attributable to Cyt oxidase. Although the photosynthetic apparatus of Chlamydomonas is present in cells grown without light, the biogenesis is enhanced with illumination, resulting in higher plastocyanin content, and therefore a corresponding increase in the quota. In iron-deficient medium, the copper quota is increased relative to iron-replete medium, coincident with a 40- to 50-fold increase in the abundance of the ferroxidase (35, 47). The impact of these physiological changes on the copper quota is visible because of the high abundance of the proteins; plastocyanin and ferroxidase are certainly visible as heavily stained bands in cell extracts (17, 48). The nutritional requirement for copper for any organism (or, by extension, for any cell within a multicellular organism) is therefore not a fixed, absolute number but rather dependent on the external conditions, physiological state, or metabolic demands on that cell.

Biomarkers for Copper Status are Organism-Specific, Dictated by Copper-Dependent Metabolic Pathways. When there is insufficient copper nutrition to support the accumulation of all cuproproteins to the maximum stoichiometry demanded by their physiology or metabolic status, a copper distribution mechanism comes into play. This mechanism relies, in part, on expression of the distributive copper transporters and chaperones. For instance, CTP1 (ortholog of S. cerevisiae Ccc2p and Arabidopsis RAN1) and ATX1 expression in inhibition of cuproprotein mRNA function in land plants is prioritized over cuproproteins required for nodule development, presumably because the loss of tyrosinase is less consequential to survival of the organism (12). Likewise, patients with weak alleles of Menkes disease (and hence less copper-deficient) show connective tissue abnormality because of lysyl oxidase deficiency but are less affected in neurological processes (54). In land plants, plastocyanin is prioritized over CuZn-superoxide dismutase because land plants do not have genetic information for Cyt c6 as a backup for plastocyanin and photosynthesis is essential for survival, whereas Fe-superoxide dismutase can cover the function of the cuproenzyme (37). The mechanisms for down-regulating the deprioritized protein vary, ranging from proteolysis of plastocyanin in Chlamydomonas to inhibition of cuproprotein mRNA function in Arabidopsis (37), but the outcome is the same.

Elemental Sparing Mechanisms May Offer a Selective Advantage. The reason that plastocyanin is dispensable under conditions of copper deficiency is because Cyt c6 is a perfect substitute with respect to bioenergetic function. Growth rates of Chlamydomonas cells in copper-replete vs. copper-deficient conditions are indistinguishable, as also are the rates of photosynthetic electron transfer (Fig. S7 and Table 3). The absence of an obvious advantage of plastocyanin raises the question of why genetic information for plastocyanin is maintained in algae and cyanobacteria. We suggest that plastocyanin offers a selective advantage as an intracellular copper store. In a competitive natural environment, where copper can become depleted during an algal bloom (exacerbated by high respiratory activity generating an oxygen-poor environment that is not conducive to copper solubility), the possibility of recycling copper from plastocyanin to Cyt oxidase would be beneficial. Cyt oxidase is an
essential enzyme because its proton-pumping function cannot be substituted by a copper-independent version. The lack of a copper-independent version is evident from the poor growth of copper-deficient Chlamydomonas cells in the dark, where they are dependent on respiration and Cyt oxidase function (Fig. S7). Despite the operation of copper sparing and salvage, Cyt oxidase-dependent (cyanide-sensitive) respiration is greatly impaired in copper-deficient Chlamydomonas [25% of Cyt oxidase-dependent respiration in copper-replete cultures (Table S1)].

In a previous work on iron allocation in cyanobacteria, the idea of “hot bunking” was promoted as a mechanism of sparing iron during a diurnal cycle (55). Saito et al. (55) suggested that intracellular iron could be shared between photosynthesis and nitrogen fixation, which are uncoupled temporally because the former is active during the day and the latter at night. In so doing, the cyano bacterium reduces its iron quota, which gives it a competitive advantage in an iron-limited ocean. However, in the study of Saito et al. (55), it was not possible to monitor recycling quantitatively, which was possible in our study because of the very small number of abundant copper proteins. It is possible that Chlamydomonas cells grown on limiting copper may also recycle the cofactor between photosynthesis and respiration on a diurnal basis, but we have not yet tested this possibility. Although plastocyanin is degraded to support Cyt oxidase accumulation, Cyt oxidase occurrence and function are not required for plastocyanin degradation. For instance, plastocyanin is degraded in the dam19 mutant (which does not accumulate Cyt oxidase) (Fig. S8).

ACKNOWLEDGMENTS. We thank Dr. Crysten Blaby-Haas for critical reading of the manuscript and Dr. Stefan Schmollinger for help with the respiration measurements shown in Fig S8. This work is supported by Grant GM42143 from the NIH.