The Involvement of a Multicopper Oxidase in Iron Uptake by the Green Algae *Chlamydomonas reinhardtii*[1]

Alexandra Herbik, Christian Bölling, and Thomas J. Buckhout*

Applied Botany, Humboldt University Berlin, Invalidenstrasse 42, 10115 Berlin, Germany

In the unicellular green algae *Chlamydomonas reinhardtii*, high-affinity uptake of iron (Fe) requires an Fe^{3+}-chelate reductase and an Fe transporter. Neither of these proteins nor their corresponding genes have been isolated. We previously identified, by analysis of differentially expressed plasma membrane proteins, an approximately 150-kD protein whose synthesis was induced under conditions of Fe-deficient growth. Based on homology of internal peptide sequences to the multicopper oxidase hephaestin, this protein was proposed to be a ferroxidase. A nucleotide sequence to the full-length cDNA clone for this ferroxidase-like protein has been obtained. Analysis of the primary amino acid sequence revealed a putative transmembrane domain near the amino terminus of the protein and signature sequences for two multicopper oxidase I motifs and one multicopper oxidase II motif. The ferroxidase-like gene was transcribed under conditions of Fe deficiency. Consistent with the role of a copper (Cu)-containing protein in Fe homeostasis, growth of cells in Cu-depleted media eliminated high-affinity Fe uptake, and Cu-deficient cells that were grown in optimal Fe showed greatly reduced Fe accumulation compared with control, Cu-sufficient cells. Reapplication of Cu resulted in the recovery of Fe transport activity. Together, these results were consistent with the participation of a ferroxidase in high-affinity Fe uptake in *C. reinhardtii*.

Copper (Cu) and iron (Fe) are essential micronutrients and function as catalysts in a variety of oxidation-reduction reactions. Because of their ability to generate free radicals, uptake of Cu and Fe into cells and their assimilation are tightly regulated to prevent both toxicity and deficiency. Carrier-mediated transport systems for Fe and Cu have been identified and characterized in several organisms. The high-affinity Fe uptake system in yeast (*Saccharomyces cerevisiae*) is composed of three enzymes, whose expression is regulated by the transcription factor AFT1 and is inversely correlated with the cellular Fe content (Yamaguchi-Iwai et al., 1995, 1996). In yeast, Fe is reduced by the Fe^{3+}-chelate reductases, FRE1 and FRE2, before transport by the high-affinity permease, FTR1 (Stearman et al., 1996; Georgatsou et al., 1997). Also participating in uptake is the multicopper oxidase, FET3 (Askwith et al., 1994). A structural and functional interaction between the multicopper oxidase, FET3, and FTR1 has been shown (Stearman et al., 1996) and confirmed by site-directed mutagenesis (Askwith and Kaplan, 1998).

Yeast cells also obtain Fe by means of a second low-affinity uptake system (Dix et al., 1994). The low-affinity Fe uptake activity is catalyzed by the proteins FET4p (Dix et al., 1994) and Smf1p and Smf2p (Liu et al., 1997). In contrast to the high-affinity uptake system, these transporter systems are composed of single proteins with broad metal specificity and preferences for Fe^{2+} over Fe^{3+}. One further transport system in yeast with homology to FET3 and FTR1 is found in the vacuole and encoded by FET5 and FTH1. This system is presumably involved in sequestration of Fe either in detoxification or as a reservoir for metals to enable the cell to grow under low Fe conditions (Stearman et al., 1996; Spizzo et al., 1997; Urbanowski and Piper, 1999).

The mechanism of Cu uptake in yeast has some similarity to the mechanism for Fe uptake. As for Fe, Cu is reduced by a Cu^{2+}-chelate reductase before uptake (Hassett and Kosman, 1995). Cu permeases are encoded by CTR1 (Dancis et al., 1994a, 1994b) and CTR3 (Knight et al., 1996), and the expression of high-affinity Cu uptake genes is regulated by Cu availability and mediated by the transcription factor MAC1 (Graden and Winge, 1997; Labbe et al., 1997; Yamaguchi-Iwai et al., 1997). The Fe^{2+} permease, FET4, also functions as a low-affinity Cu transporter and supports normal Cu uptake in yeast (Hassett et al., 2000).

Fe uptake by human cells is somewhat more complex. Transferrin-mediated Fe uptake and a further, not well-understood transferrin-independent uptake system have been described for uptake of Fe from the blood (Aisen et al., 2001). Intestinal Fe acquisition occurs at the brush boarder of the duodenal epithelial cells with subsequent export of the Fe from the epithelial cells into the blood at the basal border. Recently, McKie et al. (2001) identified a mammalian plasma membrane *b*-type cytochrome with Fe^{3+}-chelate reductase activity in the duodenal mucosa. A divalent cation transporter (DCT1p), also known as NRAMP2p and DMT1p, is responsible for the uptake

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* Corresponding author; e-mail h1131dqy@rz.hu-berlin.de, fax 49–30–20938725.

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of Fe^{2+} from the intestinal lumen (Gruenheid et al., 1995; Gunshin et al., 1997). Fe export from the duodenal epithelial cells requires the Cu-dependent ferroxidase, hephaestin (HEPH; Vulpe et al., 1999), and the permease MTP1p, which is induced under Fe deficiency (Abboud and Haile, 2000).

In Arabidopsis, a reductase activity encoded by *FRO2* (Robinson et al., 1999) and an Fe^{2+} transporter activity, encoded by *IRT1* and *IRT2* (Eide et al., 1996; Vert et al., 2001), have been shown to be involved in the Fe transport. The expression of *IRT2* was localized in external cell layers of the root subapical zone; therefore, it was suggested that this transporter was involved in the Fe uptake into the roots (Vert et al., 2001). In addition, the existence of six genes encoding NRAMP-like proteins was reported in Arabidopsis. It was shown that *AtNRAMP1* (Curie et al., 2000) and *AtNRAMP3* and *4* (Thomine et al., 2000) complemented the yeast fet3/fet4 mutant and that the *AtNRAMP1* accumulated in response to Fe deficiency, whereas *AtNRAMP3* and *4* were induced by Fe starvation. Further possible Fe transporters in Arabidopsis are encoded by eight genes homologous to *YS1* (yellow stripe), first described in maize (*Zea mays*) and shown to catalyze the Fe uptake from Fe^{3+} phytosiderophore complexes (Curie et al., 2001).

Mechanisms analogous to higher plant strategy I and II have been described in algae. Induction of a Fe^{3+}-chelate reductase and Fe uptake activity by Fe deficiency has been best characterized in *Chlamydomonas reinhardtii* (Eckhardt and Buckhout, 1998; Lynnes et al., 1998; Weger, 1999), although the enzymes catalyzing these reactions and their corresponding genes are unknown. Recently, a distinct increase of a 150-kD protein was observed by SDS-PAGE in the plasma membrane from Fe-deficient *C. reinhardtii* cells (Herbik et al., 2002). After sequencing of internal peptides of this protein by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS), homology of two peptides to expressed sequence tag (EST) clones was found (Herbik et al., 2002). Based on physiological experiments and the homology of internal peptides and deduced amino acid sequences to mammalian HEPH and multicopper oxidases in general, it was suggested that this ferroxidase-like protein (FLP) was a component of the Fe uptake complex.

In this study, we have begun a detailed examination of the function of FLP in Fe uptake. Here, we report the full-length cDNA sequence for FLP and the induction of FLP under conditions of Fe deficiency. Furthermore, we have shown that Cu and a multicopper oxidase were required for high-affinity Fe uptake. Thus, the mechanism of high-affinity Fe uptake in *C. reinhardtii* resembled that found in yeast and not that found in higher plants.

RESULTS

FLP Is a Multicopper Oxidase

Based on internal partial peptide sequences of FLP (Herbik et al., 2002), two EST clones, AV395796 and AV394010, were obtained and sequenced. Both clones were incomplete. Therefore, 5′-RACE was employed to extend the existing clones and identify a complete cDNA sequence (GenBank accession no. AY074917). Sequence analysis of the cDNA revealed an open reading frame (ORF) of 3,428 nucleotides with a potential ATG start codon at position 250. The cDNA possessed a 3′-untranslated region of about 1,000 bp, which has been found in many nuclear genes of *C. reinhardtii* (Franzén and Falk, 1992; Dinant et al., 2001), and a polyadenylation signal (TGTAA) located 13 bp upstream of the cDNA poly(A^+) sequence. An ORF encoding a protein of 1,142 amino acids with a calculated molecular mass of 131.8 kD and a pI of 4.64 was deduced from the nucleotide sequence. From SDS-PAGE, the molecular mass of the FLP protein was estimated to be 150 kD (Herbik et al., 2002). The predicted sequence contained possible glycosylation sites that may have accounted for the greater molecular mass (PROSITE; Hofmann et al., 1999). Four internal peptides sequenced in the previous study (Herbik et al., 2002) were located in the deduced polypeptide, and peptide maps obtained by MALDI-TOF MS analyses confirmed that the isolated gene coded for FLP (Fig. 1; Herbik et al., 2002). Analysis of the deduced amino acid sequence identified signature sequences for two multicopper oxidase I motifs (767–787 and 1,117–1,137) and one multicopper oxidase II motif (394–405; PROSITE; Hofmann et al., 1999). Hydrophathy analyses predicted a potential transmembrane domain for the FLP protein near to the amino terminus (amino acids 47–68; TMHMM; Moller et al., 2001).

The deduced amino acid sequence was compared with other known multicopper oxidases that have been shown to be involved in Fe transport. The predicted FLP amino acid sequence showed the greatest sequence identity and similarity to mouse HEPH (accession no. NP03447) over its entire length. The FLP and HEPH proteins were identical at 30% of their amino acid residues and displayed 45% similarity (Fig. 1). Homology of FLP was also detected to glycosylphosphatidylinositol-anchored ceruloplasmin (accession no. AAF34175, *Rattus norvegicus*, 29% identity, 43% similarity) and yeast FET3p (accession no. CAA89768, 26% identity, 45% similarity). Interestingly, a potential transmembrane domain in FLP was predicted near to the amino terminus and not at the carboxy terminus, as was the case for HEPH (Fig. 1).

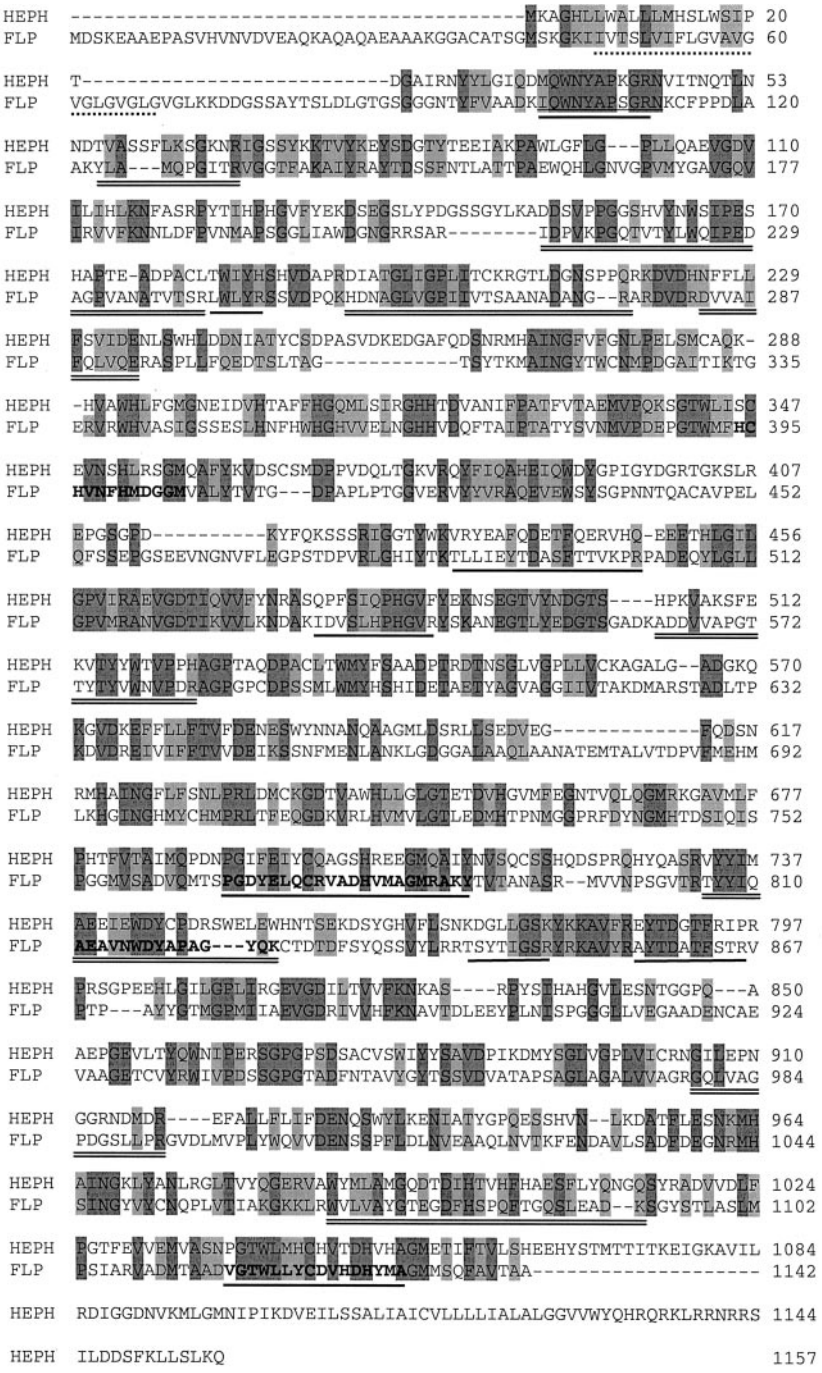

Figure 1. Comparison of the protein sequence of the FLP from *C. reinhardtii* with mouse (*Mus musculus*) HEPH. The alignment was performed using ClustalW from the EMBL database (www.ebi.ac.uk/clustalw). Dark-gray shaded amino acids represent identical residues and light gray indicates conserved substitutions in the alignment. The FLP protein (GenBank accession no. AY074917) and the HEPH protein (GenBank accession no. NP034547) show 31% sequence identity and 46% similarity. Additional features of FLP are also shown. The multicopper oxidase motif I sequences are typed in bold face and underlined, and the multicopper oxidase motif II is typed in bold face. The amino acids 47 to 68 are the putative transmembrane domain and are underlined with a dashed line, the six internal peptide sequences from FLP obtained by MALDI-TOF MS are underlined, and additional peptide maps are underlined twice.

FLP Expression Was Induced in Response to Fe Deficiency

The FLP transcript was examined by probing total RNA with two labeled DNA fragments derived from different regions of the FLP gene (Fig. 2). The FLP-A probe was derived from the 5′ region (1–786) of the FLP gene, and the FLP-B probe was located within the ORF at position 2,228 through 2,902. Compared with the control, FLP was highly expressed after 1 d of Fe deficiency as well as under combined Fe and Cu deficiency. The transcript length of about 4.6 kb agreed with the predicted ORF obtained from the cDNA sequence. After 6 d of Cu deficiency, the expression of FLP was somewhat induced compared with the control. This induction in response to Cu deficiency was distinctly less than after 1 d growth without Fe. Thus, the transcription of the FLP gene was inversely correlated to the supply of Fe and also Cu in the growth media.

Repeating the northern analysis using the FLP-B probe, a similar expression pattern was found; however, a signal at 2.5 kb in addition to the 4.6-kb signal

was prominent. These two different signals might represent alternative splicing of a single gene, specific degradation or they might represent products from two different genes. However, Southern analyses using the FLP-A probe and FLP-B probe revealed a simple pattern of hybridization (data not shown), suggesting the FLP was unlikely to be coded by multiple genes.

Induction of the Fe^{3+}- and Cu^{2+}-Chelate Reductase Activities Was Time Dependent

After 1 d of growth in Fe-deficient medium, both the Fe^{3+}- and Cu^{2+}-chelate reductases were induced to the maximum level (Fig. 3, A and B; see also Weger, 1999). However, an increase of both reductase activities was observed after 2 to 3 d of growth on Cu-depleted TAP medium. A maximum induction was reached between 6 and 8 d, although the Fe^{3+}- and the Cu^{2+}-chelate reductase activities were 10-fold lower in Cu-deficient as compared with Fe-deficient cells (Fig. 3). Previous work showed no induction of either reductase after 1 d of growth in Cu-deficient medium (Eckhardt and Buckhout, 1998). From the results shown in Figure 3, it was evident that Cu deficiency increased Cu^{2+}- and Fe^{3+}-chelate reductase activities only after prolonged growth in Cu-deficient medium. Cu deficiency was confirmed by the decrease of the Cu content in Cu-deficient cells (see below).

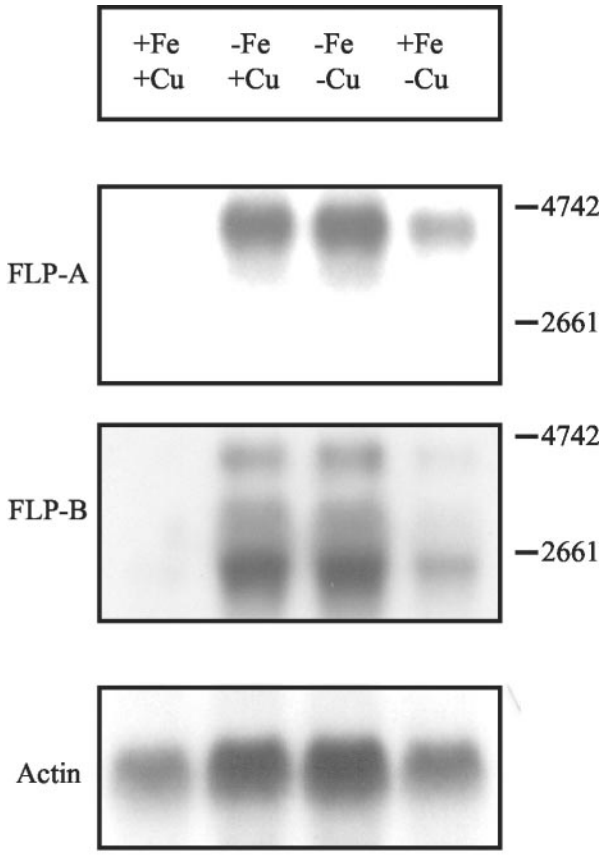

Figure 2. Expression analyses of the FLP gene in Fe- and Cu-deficient *C. reinhardtii* cells. Northern-blot analysis was performed with total RNA, and cells were grown under sufficient conditions (+), 1 d in Fe-deficient medium (−Fe), 1 d under combined Fe and Cu deficiency (−Fe/−Cu), and 6 d (+Fe/−Cu) in Cu-depleted medium. Two different probes of the FLP gene (FLP-A was derived from the 5′region and FLP-B was located at position 2,228–2,902) were used for hybridization. The 4.5-kb band corresponded to the mature FLP mRNA. A control hybridization of an actin probe using the same blots was also shown.

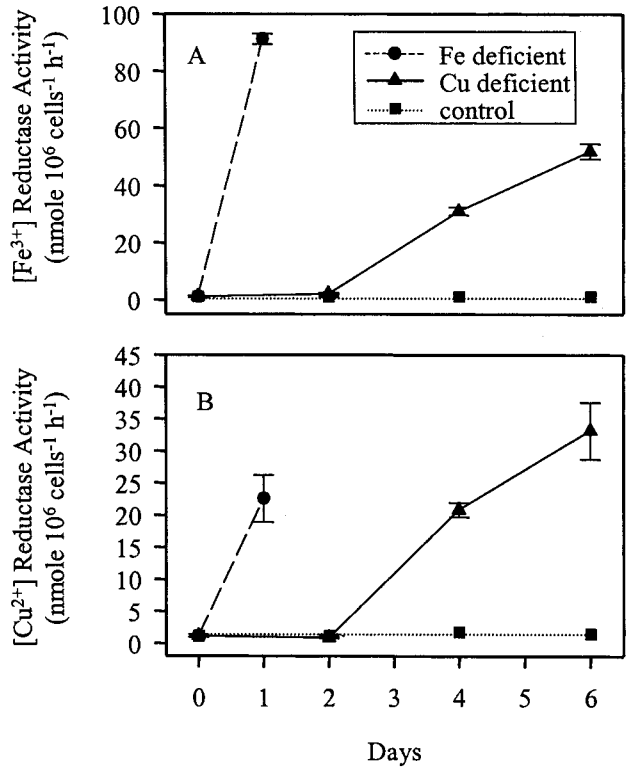

Figure 3. Fe^{3+}- and Cu^{2+}-chelate reductase activities of *C. reinhardtii*, grown in Fe- and Cu-sufficient and deficient media. The cells were grown for 1 d without Fe and up to 6 d without Cu. During the Cu-deficient growth, Tris-acetate-phosphate (TAP) medium was renewed every 2nd d and the cells were diluted to an OD_{750} of 0.2. Fe^{3+}-chelate (A) and Cu^{2+}-chelate (B) reductase activities were determined. Values represent the mean of three independent assays. SES are shown.

Fe Uptake Was Induced under Fe Deficiency and Inhibited under Cu Deficiency

If FLP is a multicopper oxidase, like FET3p or HEPH, its ability to oxidize Fe^{2+} will depend on Cu ions that activate the enzyme (Stearman et al., 1996). After 1 d of growth in Fe-deficient medium, both the Fe uptake (Fig. 4A) and Fe^{3+}- and Cu^{2+}-chelate reductase activities (Fig. 4B) were increased. Supplying Fe as Fe^{3+}-HEDTA or as Fe^{2+}-HEDTA did not significantly affect the Fe uptake activity (Fig. 4A). Importantly, cells starved of Cu and Fe showed 85%

inhibition of the Fe uptake activity compared with uptake under Fe deficiency alone. After reapplication of 6 μM Cu to cells starved of either Cu alone or both Fe and Cu, the Fe uptake and the reductase activities could be restored to the control levels or to the level found under Fe deficiency. These results clearly showed that as in yeast and mammalian cells, a Cu-dependent step was involved in the high-affinity Fe uptake in *C. reinhardtii*.

Inhibitory Effect of Tetrathiomolybdate (TTM) on Fe Uptake

If a multicopper ferroxidase were involved in high-affinity Fe transport, inhibition of this ferroxidase should abolish Fe transport. To test this hypothesis, TTM, a well-known inhibitor of multicopper oxidases, was employed (Chidambaram et al., 1984). Fe-deficient and -sufficient *C. reinhardtii* cells were pre-incubated with TTM and Fe transport activity was determined as described. The Fe uptake was slightly inhibited by the application of 25 μM TTM to Fe-sufficient and -deficient cells (Table I). A greater inhibition of the Fe uptake was obtained by the addition of 250 μM TTM, with the Fe uptake being reduced by 74% in the Fe-sufficient cells and by 89% in the Fe-deficient cells. These results were consistent with the involvement of a ferroxidase in high-affinity Fe transport.

Micronutrient Content of Cells in Response to Fe and Cu Deficiency

To confirm that the cell were deficient in Fe and Cu, Fe, Cu, Mn, and Zn content were measured in combination with the reductase activities. When grown in full medium, the cells contained 560 μg Fe, 55 μg Cu, 50 μg Zn, and 290 μg Mn g dry weight^{-1} (Fig. 5, D–G). After 1 d of Fe deficiency, the Fe and Mn content decreased by 70% and 71%, respectively. Surprisingly, Cu content increased by 39%, whereas Zn content remained unchanged. As shown, the Fe^{3+}- and the Cu^{2+}-chelate reductase activities increased under Fe deficiency (Fig. 5, B and C). Removal of Cu for 2, 4, and 6 d led to a corresponding decrease in Cu content and as for Fe deficiency, an increase in both Fe and Cu reductase activities. In contrast to the uptake experiments where the high-affinity Fe uptake was inhibited under Cu deficiency (Fig. 4A), Fe as well as Zn increased with increasing Cu deficiency (Fig. 5, D and F). We speculate that the accumulation of Fe and Zn under Cu deficiency was likely the result of uptake by a low-affinity permease.

The changes induced by Cu deficiency were reversible. Resupply of Cu to Cu-deficient cells led to a normalization of the content of all four micronutrients to the level of the control. Furthermore, resupply of Cu to Cu- and Fe-deficient cells resulted in micronutrient contents and reductase activities that were similar to those described for Fe-depleted cells. Most importantly, supplying 0.5 μM Fe to Cu depleted cells resulted in maximally induced reductase activity, reduced Fe and Cu content, and accumulation of Zn. Thus, cells grown under Cu deficiency with optimal concentrations of Fe did not accumulate Fe in contrast to Cu-deficient cells that were supplied with supra-optimal Fe, indicating the presence of a low-affinity and a Cu-dependent, high-affinity Fe uptake system in *C. reinhardtii*.

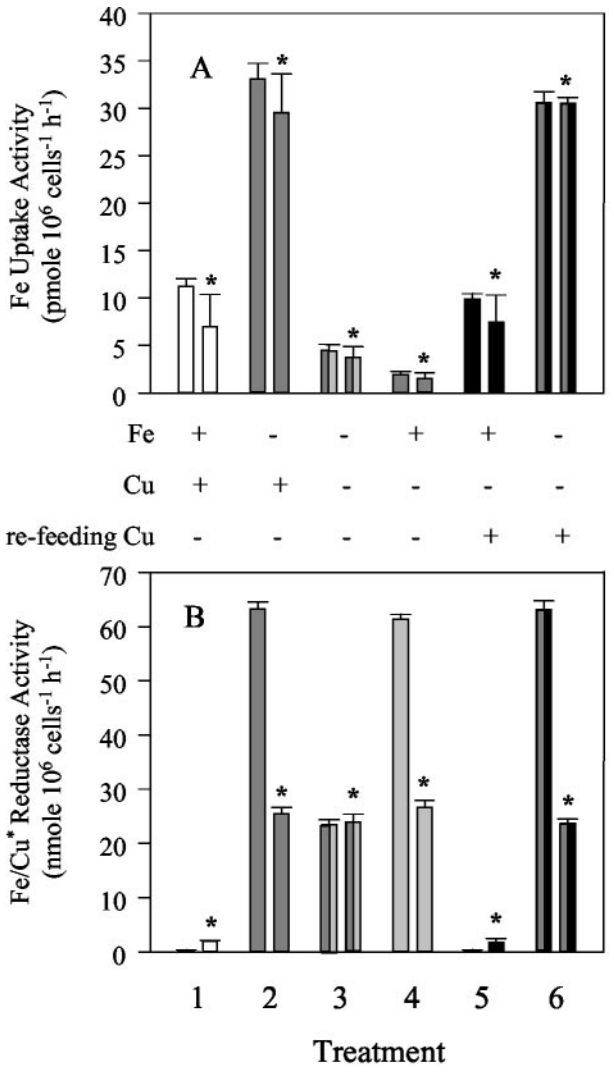

Figure 4. The influence of Cu on Fe uptake (A) and Fe/Cu reductase (B). *C. reinhardtii* cultures were subjected to different treatments: 1, control; 2, 1 d without Fe; 3, 1 d without Fe and 4 d without Cu; 4, 4 d without Cu; 5, 3 d without Cu, supplemented with Cu for the last day; and 6, 3 d without Cu and Cu resupply by concomitant remove of Fe for the last day. A, Fe was applied as Fe^{3+}-hydroxyethylenediamine triacetic acid (HEDTA), or for Fe^{2+} uptake 10 mM ascorbic acid was added (bars labeled with an asterisk). B, Fe^{3+} and Cu^{2+} (*) reductases were measured. Values represent the mean of three independent assays. SEs are shown.

Table I. *Influence of the multicopper oxidase inhibitor TTM on Fe uptake*

Twenty-five and 250 μM TTM were added to a culture containing 4.4×10^8 cells mL^{-1}. TTM was incubated for 0.5 and 4 h and the Fe uptake was measured (pmol 10^6 $cells^{-1}$ h^{-1}). These results are averages of two independent assays. Percent inhibition is shown in parentheses.

Treatment	Nutritional Status	
	+Fe	−Fe
Control	5.66 (100)	27.23 (100)
25 μM TTM, 0.5 h	5.99 (106)	21.53 (79)
25 μM TTM, 4 h	4.68 (83)	22.51 (83)
250 μM TTM, 0.5 h	1.63 (29)	10.68 (39)
250 μM TTM, 4 h	1.47 (26)	2.88 (11)

DISCUSSION

In this report, we present novel evidence linking FLP to high-affinity Fe transport and propose that FLP is a ferroxidase, functioning in the reoxidation of Fe^{2+} before its uptake into the cell. Initial evidence in support of this hypothesis comes from the deduced FLP amino acid sequence. FLP contains two multicopper oxidase I and one multicopper oxidase signature II motifs. In addition, the amino acid sequence of FLP shows the highest homology to multicopper oxidases in mammals (HEPH and ceruloplasmin) and yeast (FET3). These proteins are ferroxidases that are themselves involved in high-affinity Fe assimilation (Stearman et al., 1996; Askwith and Kaplan, 1998; Mukhopadhyay et al., 1998; Attieh et al., 1999; Vulpe et al., 1999). The involvement of FLP in Fe homeostasis is evident from the regulation of its synthesis. Both the transcription of the FLP gene and synthesis of FLP are greatly increased in Fe-deficient cells and reversed after resupply of Fe. Although we have not demonstrated the ferroxidase activity in FLP directly, we have shown previously that Fe-deficient *C. reinhardtii* cells have increased *p*-phenylenediamine oxidase activity compared with Fe-sufficient controls (Herbik et al., 2002). Furthermore, treatment of cells with TTM, an inhibitor of multicopper oxidases, results in increased Fe^{3+} reductase activity (Herbik et al., 2002) and an inhibition of high-affinity Fe uptake (Table I). These data are at the least consistent with the function of FLP as a ferroxidase.

If FLP is a component of a high-affinity Fe uptake system, one would expect it to be membrane bound. Consistent with this requirement, all data collected thus far indicate a membrane localization of FLP. FLP was initially identified in a membrane fraction highly enriched for the plasma membrane (Herbik et al., 2002), and extraction of this plasma membrane fraction with high salt and/or washing with carbonate at pH 9 does not solubilize FLP (H.I. Reinhardt and T.J. Buckhout, unpublished data). Structural analysis of the primary amino acid sequence of FLP predicts an amino terminal transmembrane domain. The presence of a single transmembrane domain is reminiscent of the predicted structure of mammalian HEPH and yeast FET3; however, in these proteins, the transmembrane domain is located on the carboxy terminus of the protein. Structural analyses also predict that the major portion of the protein is located on the outside surface of the cell (TMHMM; Moller et al., 2001). Thus, with only one transmembrane domain, FLP is unlikely to be a Fe permease. In analogy to HEPH and FET3, we propose that FLP interacts with a presently unidentified Fe transport protein to facilitate the movement of Fe across the membrane.

The proposed involvement of FLP in high-affinity Fe uptake in *C. reinhardtii* predicted a link between the Cu and Fe nutritional status of the cell and, thus, between Cu availability and Fe acquisition. Results testing this hypothesis confirmed this link and showed that Cu was necessary for high-affinity Fe transport. In Fe-deficient *C. reinhardtii*, the Fe uptake activity increased with decreasing Fe content (Fig. 4A). Removal of Cu from Fe-deficient cells resulted in an inhibition of Fe uptake activity. Furthermore, Fe uptake activity was also inhibited in Cu-deficient cells (Fig. 4A), and, finally, depletion of cellular Cu was correlated with decreased Fe in cells grown on medium containing sufficient but not supra-optimal Fe and also with the absence of high-affinity Fe transport. The reapplication of Cu to Cu-deficient cells restored the Fe uptake activity to the level of the controls. In general, whenever Cu was removed, the high-affinity Fe uptake activity was inhibited. In yeast, Cu is also required for high-affinity Fe uptake (Askwith et al., 1994; Stearman et al., 1996). Mutations either in the high-affinity Cu transporters (Dancis et al., 1994b; Yuan et al., 1995) or Cu deficiency (Askwith et al., 1994) resulted in impaired Fe uptake. Thus, in *C. reinhardtii* as in yeast, the involvement of a Cu-containing step and a multicopper oxidase activity in the high-affinity Fe uptake was apparent.

In addition to this high-affinity Fe uptake mechanism, there appeared to be additional low-affinity and low-specificity transport activities that were induced under Fe deficiency. The presence of such an activity would explain the unexpected increase in Cu content in Fe-deficient cells and the accumulation of Fe in Cu-deficient cells fed supra-optimal concentrations of Fe (Fig. 5, D and E). Long-term ^{59}Fe uptake experiments with Cu-depleted cells confirmed the accumulation of Fe when Fe was supplied at high concentrations (data not shown). The presence of low-affinity transporters has been described in yeast. For example, FET4 was shown to catalyze low-affinity Fe transport (Dix et al., 1994, 1997) and low-affinity Cu uptake (Hassett et al., 2000). It was reported that yeast strains lacking high-affinity Fe transport (Δ*fet3*) showed increased expression of FET4 and accumulated transition metals, which resulted in increased metal sensitivity (Li and Kaplan, 1998). The distinct accumulation of Fe and Zn demonstrated an accumulation of transition metals by a

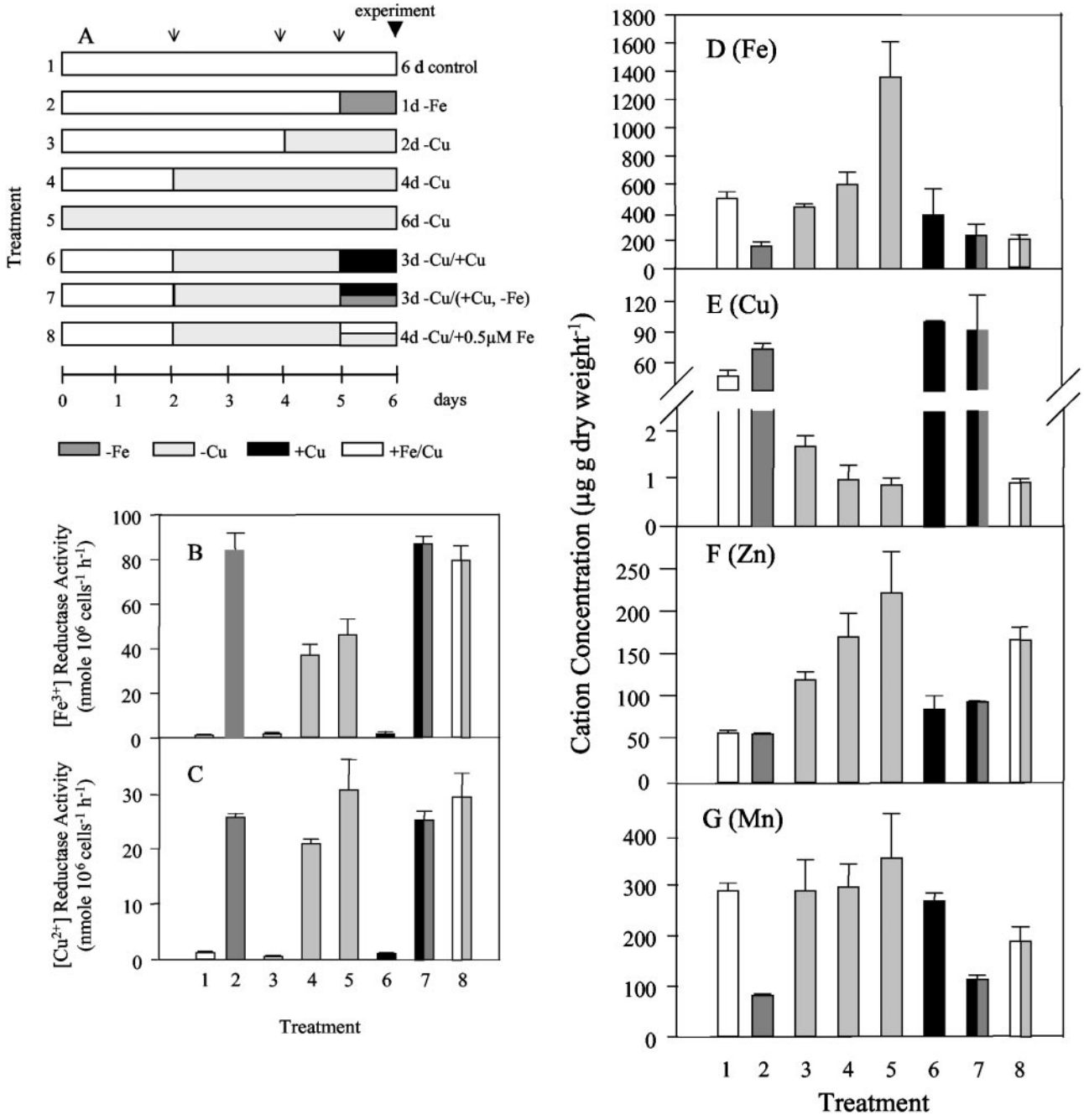

Figure 5. A through C, Total transition metal content of *C. reinhardtii* cells grown in Fe- and Cu-depleted TAP medium. A, Growth conditions up to 6 d: 1, control; 2, 1 d without Fe; 3, 2 d without Cu; 4, 4 d without Cu; 5, 6 d without Cu, 6, 3 d without Cu, supplemented with Cu for the last day; 7, 3 d without Cu and Cu resupply by concomitant removal of Fe for the last day; and 8, 4 d without Cu and reduction of the Fe supply from 20 to 0.5 μM. Arrows shown in A indicate the medium exchange and simultaneous cell dilution to an A_{750} of 0.2. B, Fe^{3+} reductase. C, Cu^{2+} reductase activities. D, Fe content; E, Cu content; F, Zn content; G, Mn content. These results are averages of two independently grown cultures.

Cu-independent system in *C. reinhardtii*. With reduction of the Fe supply from 20 to 0.5 μM in Cu-depleted cells, the high-affinity, Cu-dependent uptake activity and the low-affinity uptake activity for Fe and Cu were distinguishable. As expected under these conditions, Fe did not accumulate, and the Cu content was reduced to a minimum. However, under these same conditions, the Zn content increased by 200% (Fig. 5F). Although not characterized in this study, these results were consistent with the presence of a low-affinity Fe transport system.

Fe assimilation in *C. reinhardtii* involves an obligatory reduction of cellular Fe^{3+}-chelates, leading to chelate splitting and subsequent Fe uptake. Both

Fe^{3+}-chelate reductase and Fe uptake are induced under Fe deficiency (Eckhardt and Buckhout, 1998; Lynnes et al., 1998; Weger, 1999). What function might a combined reduction of Fe^{3+}-chelates and reoxidation of Fe^{2+} have in Fe uptake? Several authors have proposed that the ferroxidase imparts specificity and selectivity to high-affinity Fe uptake (Askwith et al., 1996; Askwith and Kaplan, 1998; Eide, 1998) The reductases FRE1 and FRE2 in yeast (Dancis et al., 1992; Hassett and Kosman, 1995) and FRO2 in Arabidopsis are nonspecific and reduce a wide range of Fe^{3+}- as well as Cu^{2+}-chelates (Robinson et al., 1999). Divalent metal carriers from plants (Eide et al., 1996; Korshunova et al., 1999; Curie et al., 2000, Rogers et al., 2000; Eckhardt et al., 2001) as well as low-affinity transporters in yeast (Dix et al., 1994, 1997) are also relatively unspecific. Thus, coupling Fe reduction to a ferroxidase might impart greater substrate specificity to Fe transport. In addition, uptake of Fe^{2+} into the cell would lead to generation of toxic hydroxyl radicals via the Fenton reaction. Reoxidation of Fe^{2+} concomitant with uptake of Fe^{3+} into the cell would avoid the production of oxygen radicals in the cell.

We have demonstrated for the first time, to our knowledge, a Cu-dependent step in high-affinity Fe uptake in *C. reinhardtii*. Furthermore, we present evidence supporting the idea that this Cu-dependent step involves the multicopper oxidase FLP as a ferroxidase. Thus, the mechanism of Fe uptake in *C. reinhardtii* resembles that in yeast and not higher plants. Studies are currently under way to test the model with loss-of-function mutants.

MATERIALS AND METHODS

Strains and Culture Conditions

The *Chlamydomonas reinhardtii* cell wall-deficient mutant strain 83/81 (cw15 mt+) was grown in Fe-sufficient and -deficient TAP medium, as described previously (Eckhardt and Buckhout, 1998). Cell density was estimated at 750 nm (Harris, 1988). Cultures were grown under Cu deficiency essentially as described by Quinn and Merchant (1998). To induce Cu deficiency, cells were collected by centrifugation, washed twice in Cu-free TAP medium, and resuspended in Cu-free TAP medium. Cells were regularly diluted to an A_{750} of 0.1 to 0.2 every 2nd d and 1 d before the experiment began. All glassware was rinsed overnight with 0.1 N HCl. Chemicals of the highest purity commercially available were used.

Fe^{3+}- and Cu^{2+}-Chelate Reductase Assays and Fe Uptake Measurements

The Fe^{3+}-chelate reductase was measured with 600 μM bathophenanthroline disulfonate as described by Eckhardt and Buckhout (1998). The Cu^{2+}-chelate reductase was measured with 100 μM bathocuproine disulfonate as described by Hill et al. (1996). In general, uptake experiments were conducted as described by Eckhardt and Buckhout (1998) with slight modifications to the uptake buffer (20 mM MES, 20 mM Na-citrate, and 2 mM K-acetate, pH 6.2) and the quench solution (20 mM MES, 20 mM EDTA, and 2 mM $CaCl_2$, pH 6.2). Before each experiment, cells were washed twice and then resuspended in uptake buffer. Five-hundred microliters of this cell suspension (2.3–2.7 $\times$ 10^6 cells mL^{-1}) was combined with 100 μL of substrate solution. When Fe^{3+} was used as a substrate, the uptake buffer contained 12 μM $FeCl_3$, 14.4 μM HEDTA, and 1,000 cpm μL^{-1} $^{59}FeCl_3$ (Amersham, Braunschweig, Germany). For Fe^{2+} uptake, 10 mM ascorbic acid was added. The reaction was stopped after 1, 2, 4.5, 9, 13.5, and 18 min by addition of 10 mL of quench solution. Cells were collected by filtration on glass fiber filters G/FC (Whatman, Kent, UK) and were washed twice with 5 mL of quench solution. The radioactivity was measured by liquid scintillation counting (Liquid Scintillation Analyzer TRI-CARB 2900TR, Hewlett-Packard, Palo Alto, CA).

Micronutrient Analysis

The cells were digested with aqua regia in a microwave oven (2 mL of HNO_3 and 6 mL of HCl; Mars 5-XP1500, CEM, Kamp-Lintfort, Germany). To verify the sample preparation, the certified reference material (BCR-CRM 414 Plankton, Institute for Reference Materials and Measurements, Geel, Belgium) was digested by the same method. The total concentrations of Fe, Mn, and Zn were determined by inductively coupled plasma (ICP) atomic emission spectrometry (Optima 3000, Perkin Elmer, Rodgau-Jügesheim, Germany). A quadrupole mass spectrometer with ICP as excitation source (ICP-MS Elan 5000; Perkin Elmer/Sciex, Rodgau-Jügesheim, Germany) was used for Cu determination. Analyses of samples followed external calibration with diluted single element and multielement standards.

Northern Blotting

Total RNA was isolated from *C. reinhardtii* cells as described by Chomczynski and Sacchi (1987). RNA was separated by agarose gel electrophoresis, blotted onto Hybond N^+ (Amersham Biosciences, Freiburg, Germany), and hybridized with the two FLP fragments that were randomly labeled with [^{32}P]dCTP (RediprimeII, Amersham Biosciences). For the reverse transcription (Omniscript, Qiagen, Hilden, Germany), the gene-specific primer GAGCCATGTTGACGGGGAAGTCC (FLP1) was used. For amplification of the 786-bp fragment at the 5′ end of the FLP gene (FLP-A), the primers CCGGGCTATCGGGAACGCCCTTTGGCG (FLP2) and CGCGAATGACCTGACCCACGGCG (FLP3) were used. For the amplification of the second probe, an 874-kb fragment at position 2,028 through 2,902 (FLP-B), the primers ATGCTGTGGATGTACCACTCC (FLP4) and CCCACCTCGGCAATGATCATGG (FLP5) were used. Blots were hybridized and the last stringent washes were at 60°C with 0.1% (w/v) SSC + 0.1% (w/v) SDS for 15 min. The blot was then subjected to autoradiography at −80°C.

5′-RACE and DNA Sequencing

To obtain the 5′ end of the cDNA for the FLP gene, 5′-RACE was performed according to the manufacture's instructions (Roche Molecular Biochemical, Penzberg, Germany). After RNA isolation, contaminating DNA was removed using DNA-free (Ambion, Austin, TX). For cDNA synthesis, Omniscript reverse transcriptase (Qiagen) and AdvantageTM-GC2 polymerase (CLONTECH, Palo Alto, CA) were used to overcome problems with the high GC content of *C. reinhardtii* sequences. Primer CCAGTCCTGCGTTGTCGTGCTTC (FLP6) was used for first strand synthesis at 42°C, primer GCCACAGGTAGGTGACTGTCTGC (FLP7) was used for the first PCR, and primer GAGCCATGTTGACGGGGAAGTCC (FLP8) was used for the nested PCR. For the cloning of the purified 1.0-kb fragment, a TA cloning kit (Invitrogen, Karlsruhe, Germany) was used. DNA was sequenced by automated ABI 373 sequencer (Applera, Weiterstadt, Germany) using Big Dye Terminator Cycle Sequencing Chemistry.

Note Added in Proof

The *C. reinhardtii* sequence, FOX1, was deposited in GenBank (accession no. AF450137) by Prof. Sabeebha Merchant (Department of Chemistry and Biochemistry, University of California, Los Angeles), and a report of this work has been recently published (S. La Fontaine, J.M. Quinn, S.S. Nakamoto, M.D. Page, V. Göhre, J.L. Moseley, J. Kropat, S. Merchant [2002] Eykaryot Cell 1: 736–757).

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