Ceruloplasmin Ferroxidase Activity Stimulates Cellular Iron Uptake by a Trivalent Cation-specific Transport Mechanism*

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The balance required to maintain appropriate cellular and tissue iron levels has led to the evolution of multiple mechanisms to precisely regulate iron uptake from transferrin and low molecular weight iron chelates. A role for ceruloplasmin (Cp) in vertebrate iron metabolism is suggested by its potent ferroxidase activity catalyzing conversion of Fe²⁺ to Fe³⁺, by identification of yeast copper oxidases homologous to Cp that facilitate high affinity iron uptake, and by studies of “acerruloplasminemic” patients who have extensive iron deposits in multiple tissues. We have recently shown that Cp increases iron uptake by cultured HepG2 cells. In this report, we investigated the mechanism by which Cp stimulates cellular iron uptake. Cp stimulated the rate of non-transferrin ⁵⁵Fe uptake by iron-deficient K₅₆₂ cells by 2–3-fold, using a transferrin receptor-independent pathway. Induction of Cp-stimulated iron uptake by iron deficiency was blocked by actinomycin D and cycloheximide, consistent with a transcriptionally induced or regulated transporter. Cp-stimulated iron uptake was completely blocked by unlabeled Fe³⁺ and by other trivalent cations including Al³⁺, Ga³⁺, and Cr³⁺, but not by divalent cations. These results indicate that Cp utilizes a trivalent cation-specific transporter. Cp ferroxidase activity was required for iron uptake as shown by the ineffectiveness of two ferroxidase-deficient Cp preparations, copper-deficient Cp and thiomolybdate-treated Cp. We propose a model in which iron reduction and subsequent re-oxidation by Cp are essential for an iron uptake pathway with high ion specificity.

Iron is important in many biological processes because it is an ideal O₂ carrier and because it can function as a protein-bound redox element. Iron deficiency is common worldwide and in infants can cause severe neurological deficit (1). In contrast, iron in excess of cellular needs is toxic and dietary overload or hereditary hemochromatosis (HH) leads to tissue iron deposits in multiple tissues. We have recently shown that Cp increases iron uptake by cultured HepG2 cells. Cp stimulated the rate of non-transferrin ⁵⁵Fe uptake by iron-deficient K₅₆₂ cells by 2–3-fold, using a transferrin receptor-independent pathway. Induction of Cp-stimulated iron uptake by iron deficiency was blocked by actinomycin D and cycloheximide, consistent with a transcriptionally induced or regulated transporter. Cp-stimulated iron uptake was completely blocked by unlabeled Fe³⁺ and by other trivalent cations including Al³⁺, Ga³⁺, and Cr³⁺, but not by divalent cations. These results indicate that Cp utilizes a trivalent cation-specific transporter. Cp ferroxidase activity was required for iron uptake as shown by the ineffectiveness of two ferroxidase-deficient Cp preparations, copper-deficient Cp and thiomolybdate-treated Cp. We propose a model in which iron reduction and subsequent re-oxidation by Cp are essential for an iron uptake pathway with high ion specificity.

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sequence homology to human ceruloplasmin (Cp), and like Cp is a copper protein with a ferroxidase activity that catalyzes conversion of ferrous to ferric iron.

The similarities between Fet3p and Cp suggest a possible role for the latter in vertebrate cell iron uptake. We have shown recently that Cp increases 55Fe uptake by iron-deficient HepG2 cells by a transferin receptor-independent pathway (23). Furthermore, iron deprivation transcriptionally induces Cp synthesis by HepG2 cells (23), and Fet3p synthesis in yeast (20, 21). Thus maintenance of iron homeostasis in liver (and possibly other tissues) during periods of iron deficiency may be an important physiological function of Cp. The recent observation that “aceruloplasminemia” patients with Cp gene defects have massive iron deposits in liver, pancreas, brain, retina, and other tissues indicates an important role for Cp in iron homeostasis in vivo (22, 24, 25).

In this report we investigate the molecular mechanism by which Cp increases iron uptake by erythroleukemic K562 cells. Our results show that Cp-stimulated iron uptake requires Cp ferroxidase activity and utilizes a novel, trivalent cation-specific transport pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Purified human Cp was obtained from Calbiochem (La Jolla, CA). Cp homogeneity was verified by an absorbance ratio (610 nm/280 nm) higher than 0.045, and by SDS-PAGE and Coomassie Blue staining. Densitometric analysis showed that about 80–95% was present as the intact, 132-kDa form of Cp. 59FeCl3 (20 μM/μg), 59FeCl2 (19.22 μM/μg), and 54CrCl3 (789 μM/μg) were from NEN Life Science Products Inc. (Boston, MA). Rabbit anti-human apotransferrin antibody was from Accurate (Westbury, NY), anti-human transferrin receptor mouse monoclonal antibody (H86.4) was from Zymed Laboratories Inc. (South San Francisco, CA), and rabbit anti-human ferritin IgG was from Boehringer Mannheim. Nitrocellulose filters were from Millipore (Bedford, MA). Ammonium tetrathiomolybdate was from Sigma-Aldrich (Milwaukee, WI), and human apotransferrin, horse spleen apoferritin, reduced glutathione (GSH), cycloheximide, nitritolriacetic acid (NTA), bathophenanthroline disulfonate, and other assay reagents were from Sigma.

**Measurement of 59Fe Uptake by K562 Cells**—Human erythroleukemic K562 cells (ATCC, Rockville, MA) were grown in iron-free RPMI 1640 medium supplemented with 10% fetal bovine serum. The cells were made iron-deficient by resuspension in serum-free RPMI 1640 medium containing bathophenanthroline disulfonate (100 μM) for 4–8 h. The cells were washed twice with phosphate-buffered saline and once with RPMI 1640 medium, and then resuspended in the same medium.

**Preparation of Apoceruloplasmin and Reconstitution of Holoceruloplasmin**—Apoceruloplasmin (apoCp) was prepared by removal of Cp copper by complexing with cyanide under reducing conditions as described by Mucsi et al. (27). Cp was dialyzed at 4 °C against 100 mM sodium acetate buffer (pH 5.9) containing 10 mM ascorbate under anaerobic conditions achieved by continuous bubbling with N2 gas. Dialysis was continued until the “blue” copper complexes were reduced as shown by complete decolorization of the sample. Potassium cyanide (50 mM) was added and the dialysis continued for 5 h. The copper-cyanide complex and excess cyanide were removed by dialysis against 100 mM sodium acetate containing 1 mM cyanate, and then by overnight dialysis against buffer containing 50 mM MOPS, 150 mM potassium chloride (pH 7.0). Inactivation was verified by an “in-gel” ferroxidase assay (see below).

**Measurement of Cp Ferroxidase Activity**—Cp ferroxidase activity was measured by a modification of the method of Schen and Rabinovitz (30). In brief, Cp or modified Cp was subjected to electrophoresis on a 1% agarose gel (Seakem Gold, FMC, Rockland, ME) under nondenaturing conditions. After transfer to an Immobilon-P membrane, the blot was incubated with 0.08% ferrous sulfate in acetic acid buffer (pH 5.7), for 30 min at 37 °C, followed by incubation with 1% potassium ferrocyanide in 0.1 M HCl. Ferric iron (formed by Cp-catalyzed oxidation of ferrous ion) was detected by the rapid formation of a “Prussian blue” band which was stable for at least 24 h.

**RESULTS**

**Characterization of Cp-stimulated Iron Uptake by K562 Cells**—We have previously shown that Cp stimulates high affinity iron uptake by iron-deficient HepG2 cells (23). For a more detailed analysis of the mechanism of Cp-stimulated iron uptake, we used human erythroleukemic K562 cells since the transferrin-dependent and -independent uptake pathways are well characterized (10). Unlike HepG2 cells which secrete Cp in an amount sufficient to promote iron uptake (23), K562 cells do not secrete Cp (or transferrin, Ref. 31) thereby permitting more precise control of conditions. Cp-stimulated 59Fe-NTA uptake by iron-deficient K562 cells by about 2–3-fold (Fig. 1A). Half-maximal stimulation was at about 10 μg/ml Cp and maximal uptake was at about 30 μg/ml, a level much lower than the unevoked human plasma concentration of 300 μg/ml. The stimulation of iron uptake by Cp was observed in iron-deficient cells but not in iron-sufficient cells (Fig. 1B), a finding consistent with studies of HepG2 cells (23). Cp-stimulated iron uptake by decreasing the apparent Km of 59Fe-NTA uptake from about 2 μM to about 0.5 μM, without changing the maximum uptake rate (Fig. 1B). This result indicated that the number of active transporters was not altered by Cp, but rather that the affinity of the transporter for iron ions increased or that the amount of substrate flux was increased by Cp.

Several control experiments were done to show that Cp stimulated iron entry into the cell, and thus exclude the possibility that Cp increased extracellular binding of iron to the cell surface (e.g. by ferroxidase-dependent conversion of soluble Fe3+ to insoluble Fe3+). First, we measured iron uptake using a
procedure specifically designed to remove complexes of surface-bound iron (32). According to this method, cells are rinsed with a solution containing 5 mM sodium dithionite to reduce Fe$^{3+}$ to Fe$^{2+}$ and with 5 mM bathophenanthroline disulfonate to specifically chelate Fe$^{2+}$. This procedure gave results essentially identical to those obtained with the normal rinse solution containing 10 mM EDTA. Cp increased iron uptake by 2–3-fold in iron-deficient K562 cells when measured using either the "reducing" wash or the normal wash (data not shown). To verify iron internalization, intracellular and membrane-bound iron was measured. K562 cells were incubated with $^{59}$Fe-NTA, the cells were lysed, and the membrane and cytosol fractions isolated by centrifugation. In this experiment Cp stimulated about a 2-fold increase in radioactivity measured in intact (and iron-deficient) cells, and nearly all of this increase was accounted for by $^{59}$Fe in the cytosolic fraction (Fig. 1C); 10% or less of the total cell radioactivity was bound to membranes under any condition. To verify the intracellular localization of $^{59}$Fe, incorporation into protein was determined by SDS-PAGE and autoradiography. Radiolabeled protein was not detected in the membrane fraction of any samples (Fig. 1, D and E). However, the cytosolic fraction exhibited a single major $^{59}$Fe-labeled band that co-migrated with ferritin (Fig. 1F). Identification of the $^{59}$Fe-labeled band as ferritin was confirmed by immunodepletion with anti-ferritin IgG, but not by a nonspecific antibody (Fig. 1F). Cp increased incorporation of $^{59}$Fe into ferritin in lysates made from iron-deficient cells by about 90%; equal protein loading of the gel lanes was shown by Coomassie protein staining (Fig. 1G). The effect of Cp on iron uptake in K562 cells. A, $^{55}$Fe-NTA (1.4 μM) was incubated with iron-deficient K562 cells in the presence of purified human Cp for 20 min at 25 °C, and $^{55}$Fe uptake was measured by a filtration assay. B, iron-deficient (Fe-def.; circles) or iron-sufficient (Fe-suff.; triangles) K562 cells were incubated with $^{55}$Fe-NTA in the presence (closed symbols) or absence (open symbols) of Cp (30 μg/ml) for 20 min at 25 °C and $^{55}$Fe uptake was measured. C, iron-deficient K562 cells were incubated with $^{59}$Fe-NTA (1.4 μM) in the absence (open hatched bars) or presence (black bars) of Cp. The cells were lysed, and the membrane fraction was separated from the cytosol-containing fraction by centrifugation. Amounts of $^{59}$Fe were determined by γ-ray scintillation counting. D, aliquots of membranes (20 μg of protein) described in C were subjected to SDS-PAGE under non-reducing conditions and autoradiography. E, Coomassie Blue stain of the gel in D. F, aliquots of lysates (20 μg of protein) described in C were subjected to SDS-PAGE under non-reducing conditions and autoradiography. As a control, ferritin was removed from the cytosolic fraction by immunoprecipitation (IP) with anti-ferritin IgG (anti-ferr.; 1:1000) or nonspecific IgG (1:1000). G, Same as F but the gel was stained with Coomassie Blue. The position of ferritin was determined using unlabeled ferritin as a standard (Ferr. std.; 25 μg) and is indicated by an arrow. H, K562 cells were treated with medium only (open hatched bars), 10 μg/ml actinomycin D (black bars), or 10 μg/ml cycloheximide (closely hatched bars) for 1 h and then made iron-deficient by treatment with bathophenanthroline. The cells were incubated for 20 min in the presence or absence of 30 μg/ml Cp and $^{55}$Fe uptake was measured. I, K562 cells were preloaded with $^{59}$Fe-NTA (11.4 μM) for 30 min at 25 °C. Cells were then washed and reincubated for 20 min with apotransferrin (apoTf; 30 μg/ml), NTA (0.2 mM), or Cp (30 μg/ml) as indicated. Release of $^{59}$Fe into the medium was measured.
Blue stain (Fig. 1G). These results demonstrate that the increase in iron associated with cells in the presence of Cp is due to cellular uptake and entry into a metabolically active intracellular iron pool.

The finding that Cp did not stimulate iron uptake by iron-sufficient cells (Fig. 1B) suggested that a factor required for uptake, e.g. the transporter itself, was induced or activated by iron deficiency. To test whether this factor must be synthesized \textit{de novo}, K562 cells were treated with actinomycin D or cycloheximide prior to iron depletion. Cp-stimulated iron uptake was completely blocked by both inhibitors suggesting transcriptional up-regulation of an iron transporter (or a regulatory protein) by iron deficiency (Fig. 1H). To determine whether Cp stimulates iron efflux as well as influx, K562 cells were pre-loaded with $^{55}$Fe-NTA, and Cp-stimulated iron release was measured. Under these conditions, Cp did not enhance iron release (Fig. 1I). The addition of apo-transferrin or NTa to the medium as Fe$^{3+}$ acceptors did not alter this result. This result was consistent with our previous findings in HepG2 cells (23), but not with results of Young et al. (33) who reported a small Cp-stimulated increase in iron efflux from HepG2 cells. The difference may be due to experimental conditions, e.g. our methods measure the effects of Cp on initial rates whereas the 18-h efflux intervals of Young et al. (33) may reflect equilibrium processes shifted by Cp.

\textit{Transport Mechanism of Cp-stimulated Iron Uptake—}The conversion of Fe$^{2+}$ to Fe$^{3+}$ by Cp ferroxidase activity is thought to facilitate the binding of iron to apotransferrin (34). It is thus possible that Cp stimulates cellular iron uptake by stimulating the formation of Fe$_2$-transferrin which delivers Fe$^{3+}$ by transferrin receptor-mediated endocytosis. Although K562 cells are not known to secrete transferrin (31), this pathway was tested by addition of a rabbit polyclonal anti-human transferrin antibody (not shown). These results show that Cp-stimulated iron uptake was transferrin receptor-independent, but did not exclude the possibility that other receptor-mediated endocytic processes were involved, e.g. endocytosis of the putative Cp receptors (35, 36) or other iron-protein receptors. The lysosomotropic inhibitor, amantadine, was used to inhibit receptor-mediated endocytosis (37). Amantadine had essentially no effect on Cp-stimulated $^{55}$Fe uptake, but completely blocked $^{55}$Fe uptake mediated by transferrin (Fig. 2A). Essentially identical results were obtained using a monoclonal anti-human transferrin receptor antibody (not shown). These results show that Cp-stimulated iron uptake was transferrin receptor-independent, but did not exclude the possibility that other receptor-mediated endocytic processes were involved, e.g. endocytosis of the putative Cp receptors (35, 36) or other iron-protein receptors. The lysosomotropic inhibitor, amantadine, was used to inhibit receptor-mediated endocytosis (37). Amantadine had essentially no effect on Cp-stimulated $^{55}$Fe-NTA uptake, but was a very effective inhibitor of transferrin-mediated iron uptake (Fig. 2B). Comparable results were obtained using ammonium chloride (38) to inhibit receptor-mediated endocytosis (data not shown).

\textit{Cation Specificity and Role of Ferroxidase Activity in Cp-stimulated Iron Uptake—}The cation specificity of Cp-stimulated iron transport was investigated. Multiple divalent cations, including Ca$^{2+}$, Mg$^{2+}$, Cu$^{2+}$, or Zn$^{2+}$ (and Mn$^{2+}$ and Co$^{2+}$ not shown), added at a 70-fold molar excess did not substantially compete for Cp-stimulated $^{55}$Fe uptake (Fig. 3). In the same experiment Fe$^{2+}$ (FeCl$_3$ plus ascorbic acid) in the presence of Cp completely suppressed uptake. This result suggests that Cp stimulates a transporter with absolute specificity for Fe$^{2+}$ or a trivalent cation transporter; the latter is consistent with a requirement for ferroxidase activity of Cp.

The role of Cp ferroxidase activity was examined using ferroxidase-defective Cp. We took advantage of the ability of thiomolybdate to bind copper and irreversibly inhibit Cp ferroxidase activity (29). Treatment of Cp with ammonium tetrathiomolybdate completely blocked its iron uptake activity (Fig. 4A). Thiomolybdate did not inhibit iron uptake by inactivation of the cellular transport pathway since the addition of untreated Cp to cells incubated with thiomolybdate-treated Cp fully restored iron uptake. The inactivation by thiomolybdate treatment was confirmed by an in-gel assay for ferroxidase activity (Fig. 4B). The integrity of apoCp was tested by SDS-PAGE analysis and Coomassie Blue staining which revealed a
Ceruloplasmin Ferroxidase Activity Stimulates Iron Uptake

**Fig. 4. Requirement for ferroxidase activity in Cp-induced iron uptake.**
A, Cp was reacted with ammonium tetrathiomolybdate (CpTM) as described under “Experimental Procedures.” Iron-deficient K562 cells were incubated with 5 nM Fe-NTA (1.4 μM) in the presence of Cp (30 μg/ml), CpTM (30 μg/ml) plus Cp (30 μg/ml; 1 × Cp), or plus Cp (150 μg/ml; 5 × Cp), or with thiomolybdate alone (2.35 μM). Iron uptake was measured after 20 min. B, ferroxidase activity of 5 μg of Cp and CpTM were measured by an in-gel assay. C, Cp and CpTM (5 μg) were subjected to SDS-PAGE and Coomassie Blue staining. D, apoCp was prepared by complexing copper with cyanide as described under “Experimental Procedures.” Cp was reconstituted with copper by incubation with CuGSH. Iron-deficient K562 were incubated with 55Fe-NTA (1.4 μM) in the presence of medium only, Cp, apoCp, or Cp, and then 55Fe uptake was measured. E, ferroxidase activity of 5 μg of Cp, apoCp, and Cp, was measured by an in-gel assay. F, integrity of Cp, apoCp, and Cp, was examined by SDS-PAGE (4–12% gel) and Coomassie Blue staining.

**Fig. 5. Effect of trivalent cations on Cp-stimulated iron uptake.** A, iron-deficient K562 cells were incubated with 1.4 μM 55Fe-NTA, 1 mM ascorbate, and 0–100 μM FeCl3 (●), AlCl3 (△), or CrCl3 (△), or GaCl3 (○) in the presence of 30 μg/ml Cp. In a negative control experiment, cells were similarly incubated in the absence of Cp (×). 55Fe uptake was measured after 20 min. B, ferroxidase activity of Cp (5 μg) incubated with 0–100 μM CrCl3 for 30 min at 37 °C was measured by in-gel assay. C, same as in B but Cp incubated with 0–100 μM AlCl3, D, same as in B but Cp incubated with 100 μM GaCl3.
cells. Second, $^{51}$Cr uptake was inhibited by Fe$^{3+}$ (and by Cr$^{3+}$ and Al$^{3+}$), and by Fe$^{2+}$ plus Cp, but not by Fe$^{2+}$ alone (Fig. 6). Cp did not increase $^{51}$Cr uptake, thus providing additional evidence that Cp does not directly activate the transporter. Together these results show that the transporter mediating Cp-stimulated Fe$^{3+}$ uptake is a high affinity trivalent cation transporter.

**DISCUSSION**

Our results show that Cp stimulates cellular iron uptake by ferroxidase-dependent conversion of Fe$^{2+}$ to Fe$^{3+}$ and transport by a trivalent cation-specific transporter. This uptake mechanism recalls the newly elucidated iron uptake pathway in *S. cerevisiae* and *S. pombe* shown in Fig. 7A (24). According to this model the yeast ferrireductase Fre1p/Fre2p initiates the uptake pathway by one-electron reduction of Fe$^{3+}$ to Fe$^{2+}$. The reduced metal is reoxidized to Fe$^{3+}$ by Fet3p (or Fio1p), a Cp homologue with ferroxidase activity. Finally, Fe$^{3+}$ is transported into the cell by Ftr1p (or Fip1p), a six-spanning membrane transporter that physically interacts with Fet3p (Fio1p). These yeast proteins are all transcriptionally up-regulated during iron deficiency by activation of the Aft1p transcription factor (40). By analogy we propose a model in which Cp is positioned in the center of an analogous iron transport pathway for mammalian cells (Fig. 7B). In this model extracellular iron is first reduced by a surface ferrireductase, re-oxidized by Cp, and finally transported into the cell by a trivalent cation-specific transporter. The evidence for the mammalian pathway elements, and the similarities and differences with their yeast counterparts, are addressed below.

The requirement for a surface ferrireductase activity for transferrin-independent iron uptake has been shown for human HeLa (16), HepG2 (9), and K562 cells (15). Our studies have not addressed the role of ferroxidase activity in Cp-stimulated iron uptake; however, the requirement for the reductant ascorbate (not shown) is consistent with an important role of a ferrireductase under physiological conditions (i.e., in the absence of exogenous reductant). The identity of the mammalian ferrireductase is not known, but the phagocyte NADPH oxidase complex may be involved since Fre1p has partial sequence homology to its gp91-phox subunit (19).

Our results show that Cp stimulates $^{50}$Fe uptake by iron-deficient K562 cells by up to 2-3-fold. An absolute requirement for ferroxidase activity for Cp-stimulated iron uptake was shown using two ferroxidase-deficient preparations: thiomylobate-treated Cp and copper-depleted apoCp. This part of the pathway is analogous to the yeast pathway since Fet3p (and Fio1p), a protein with some sequence homology to Cp, has been implicated in high affinity iron uptake (20). Fet3p, like Cp, is a multicopper oxidase with O$_2$-dependent ferroxidase activity (21). A requirement for Fet3p ferroxidase activity in iron uptake has been suggested by the correlation between ferroxidase activity (and Fet3p expression) and iron uptake (20, 22). A recent study has shown directly, by site-directed mutagenesis, that the ferroxidase activity of Fet3p is required for high affinity iron uptake by yeast (41). A similarity between the yeast and mammalian systems is found in the transcriptional up-regulation by iron deficiency of Fet3p in *S. cerevisiae* (20) and Cp in HepG2 cells (23). K562 cells do not produce Cp, indicating tissue-specific expression (not shown). A species-specific difference is seen in the cellular localization of the ferroxidase. In yeast, Fet3p is membrane-bound and physically interacting with a membrane transporter (21). In contrast, mammalian Cp has a signal sequence and is efficiently secreted. This difference may reflect the paracrine nature of iron homeostasis in mammalian systems that is absent in yeast. The spatial relationship between Cp and the trivalent cation transporter is not known. One possibility is that Cp transiently associates with the transporter to increase the efficiency of Fe$^{3+}$ delivery. The identification of putative Cp-binding proteins on the surface of multiple cell types (36), which are up-regulated by iron deficiency (42), is consistent with this mechanism. Alternatively, Fe$^{3+}$ provided by Cp may be delivered to the transporter by diffusion.

The iron specificity of the mammalian non-transferrin iron uptake pathway(s) is uncertain, due in part to differences between the cells and uptake assays used in various laboratories. Our experiments show that the Cp-stimulated iron transporter in K562 cells is trivalent cation-specific based on the lack of inhibition by divalent cations, the inhibition by trivalent cations, the transport of $^{51}$Cr, and the requirement for ferroxidase activity in iron transport. Others have suggested that Fe$^{2+}$ is
the principle transport form based on the requirement for reducing agents and inhibition by Fe$^{3+}$-specific chelators (9). However, in light of recent studies showing the participation of both reduced and oxidized iron in the transport pathway, these approaches do not give specific information on the actual ion transported. Further evidence for Fe$^{3+}$ transport has been provided by studies showing that non-transferrin iron uptake is blocked by divalent cations (43). However, studies in K562 cells show an inability of divalent cations to compete, a finding in agreement with our own (10). Two previous reports suggest that Ga$^{3+}$ and other trivalent cations regulate iron uptake (44, 45), but there is no direct evidence for iron uptake via a trivalent cation-specific pathway. To our knowledge the ion specificity of the yeast iron transporters Ftr1p and Fio1p have not been reported.

The identity of the transporter facilitating Cp-stimulated iron uptake is not known. Identification of the specific genes encoding the two yeast transporters has not been helpful since homologous mammalian cDNA's have not been identified. Several mammalian iron transporters have recently been discovered and are pathway candidates. One such iron transporter is DCT1, a member of the Nramp gene family (14). However, divalent cations are efficiently transported by DCT1 suggesting it is not linked to Cp-stimulated transport. A possible role for SFT, an iron transporter cloned from phorbol ester-treated K562 cells, is supported by the fact that the SFT-mediated iron transport is not inhibited by most divalent cations. However, its localization in recycling endosomes, and the stimulated uptake of both transferrin-bound iron and non-transferrin iron uptake, indicate a role in intracellular iron transport (13).

The presence of iron reduction and oxidation steps in a single pathway is surprising. In fact, early models of the yeast uptake pathway described an extracellular ferrireductase and an intracellular ferroxidase, thus avoiding a “futile” cycle (20, 46). This model has since been modified by biochemical studies, and the ferrireductase domain of Fe3p, like Cp, is extracellular (21). The function of the ferrireductase is most likely to release soluble Fe$^{2+}$ from Fe$^{3+}$ complexes (Fe$^{3+}$-NTA in our experiments, possibly non-transferrin bound iron in vivo). The function of the ferrireductase is clearly to convert the solubilized Fe$^{2+}$ to the Fe$^{3+}$ form that is recognized by the downstream divalent cation transporter. The advantage of a two-step pathway of iron transport rather than a simpler one-step pathway of direct Fe$^{2+}$ transport via a divalent cation-specific transporter is uncertain. One possibility is that the two-step pathway gives higher cation specificity. For example, a trivalent cation-specific transporter could greatly improve specificity because Fe$^{3+}$ will compete successfully with the relatively rare trivalent cations found in tissues. In the case of a divalent cation transporter, Fe$^{2+}$ would have a much more difficult task competing with abundant ions such as Mg$^{2+}$, Zn$^{2+}$, etc. According to this proposed mechanism, the ferrireductase activity improves iron solubility and the ferroxidase activity improves transport specificity.

The precise role of Cp in iron homeostasis in vivo is poorly understood. The data presented here and elsewhere suggest that alterations in plasma Cp concentration or activity may alter iron homeostasis, and could contribute to human disease. The finding of hemochromatosis in aceruloplasminemia patients is generally considered as evidence that the normal function of Cp is to mediate iron efflux from cells and tissues (47, 48). However, these studies may also be consistent with a role of Cp in cellular iron uptake. For example, iron taken into cells by the Cp-mediated pathway might reduce subsequent iron uptake more efficiently than iron taken in by other pathways. In this case, Cp may act in effect as a negative regulator of iron uptake, and iron-loading would occur in its absence. Alternatively, the defect in Cp-mediated iron influx may lead to extracellular accumulation of an iron form to a concentration that permits nonspecific, low affinity uptake by cells, thus explaining the observed tissue overload. A more detailed understanding of the function of Cp in cellular iron metabolic pathways will put us in a better position to answer questions on the role of Cp in iron homeostasis in physiological and pathological states.

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