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Chloride is an allosteric effector of copper assembly for the yeast multicopper oxidase Fet3p: An unexpected role for intracellular chloride channels

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ABSTRACT ***GEF1* is a gene in *Saccharomyces cerevisiae*, which encodes a putative voltage-regulated chloride channel. *gef1* mutants have a defect in the high-affinity iron transport system, which relies on the cell surface multicopper oxidase Fet3p. The defect is due to an inability to transfer Cu^+ to apoFet3p within the secretory apparatus. We demonstrate that the insertion of Cu into apoFet3p is dependent on the presence of Cl^-. Cu-loading of apoFet3p is favored at acidic pH, but in the absence of Cl^- there is very little Cu-loading at any pH. Cl^- has a positive allosteric effect on Cu-loading of apoFet3p. Kinetic studies suggest that Cl^- may also bind to Fet3p and that Cu^+ has an allosteric effect on the binding of Cl^- to the enzyme. Thus, Cl^- may be required for the metal loading of proteins within the secretory apparatus. These results may have implications in mammalian physiology, as mutations in human intracellular chloride channels result in disease.**

Voltage-regulated chloride channels (CLCs) are a family of proteins widely distributed in eukaryotes. There are multiple human CLC genes, many of which show tissue-specific expression (1). A mutation in *CLC1* is responsible for myotonic dystrophy (2, 3), whereas defects in *CLC5* and *CLCKB* result in kidney diseases (4). Some CLCs are located on the cell surface, where they mediate the vectoral transport of Cl^- (5). Other CLC family members have a subcellular rather then a surface localization (6). Recently *CLC5*, which is highly expressed in kidney and is encoded by the gene responsible for Dent's disease, was shown to be localized in endocytic vesicles (7). One of the hallmarks of this disorder is defects in calcium homeostasis as well as an increased secretion of proteins. The mechanism(s) by which a dysfunctional subcellular CLC leads to these pathophysiological features is unclear. It has been suggested that CLCs maintain the electrochemical potential of vesicles such as endosomes and lysosomes (8). The absence of a counter anion such as Cl^- would limit the concentration of cations, such as hydrogen, within vesicles, disrupting pH and vesicle function.

Studies of the yeast CLC gene, *GEF1*, have suggested that intracellular CLCs play a role in vesicular cation homeostasis (9). Yeast *gef1* mutants show an inability to grow on medium containing nonfermentable substrates and on low-iron glucose-containing medium (10). These phenotypes are ascribed to a defect in the Cu-loading of apoFet3p (9). High-affinity plasma membrane iron transport in *Saccharomyces cerevisiae* requires a multicopper oxidase, Fet3p, which converts Fe^{2+} to Fe^{3+}, and a transmembrane iron permease, Ftr1p (11, 12). Cu-loading of Fet3p occurs in a post-Golgi vesicle and is meditated by the action of a P-type ATPase, Ccc2p, which is an orthologue of the human Menkes'/Wilson's disease gene products (13). Gef1p is localized to the same Golgi or trans-Golgi compartment as Ccc2p (9). Gaxiola *et al.* (9) suggested that the defect in Fet3p activity in *gef1* mutants results from a defect in vesicular Cu homeostasis. In the absence of a counter ion such as Cl^-, cation transport would result in an unfavorable electrochemical potential that may affect Cu-loading of apoFet3p by limiting the transport of H^+ or Cu^+. We confirm that defective *gef1* function leads to a Cu-deficient Fet3p. We also report that Cl^- is an allosteric effector of the Cu-loading of Fet3p. Without Cl^-, apoFet3p is not Cu-loaded and fails to function as a ferroxidase. We suggest that Cl^- provided by CLCs may be required for the metallation and or activity of vesicular enzymes.

METHODS AND MATERIALS

Cells and Medium. The mutant with the *gef1–2* allele, whose parental strain was DY1457, was identified by its inability to grow on low-iron medium. It was obtained as part of a streptonigrin screen for mutants that were defective in iron metabolism (*14*). The *gef1* deletion strain (Δ*gef1*) was generated by using the PCR-based disruption protocol described by Amberg *et al.* (15). The specific primers used were GEF1FWD, 5′-AGTGGATCCAATTATTTGACACTAATGCCAACA-ACTTAT-3′; GEF1KO2, 5′-GTCGTGACTGGGAAAACC-CTGGCGCCTCTGACAATGACCTGTTTT-3′; GEF1KO3, 5′-TCCTGTGTGAAATTGTTATCCGCTTCTAACGAA-GAGTTGGGACATCGT-3′; and GEF1REV, 5′-ATC-GAATTCTATAACGTTACCATTTCGATTAGTGGT-3′. Use of these primers deleted 1.7 kb of the *GEF1* gene. The template vector for PCR of the auxotrophic marker was pJJ282 bearing the *LEU2* gene. Strain MM19 has a mutation in the *ccc2* gene and a deletion in *fet4* (16). The presence of the *fet4* deletion results in the loss of the low-affinity iron transport system, increasing cellular iron deprivation, which results in an increased expression of *FET3* mRNA and of apoFet3p. Thus, this mutant does not need to be grown in low-iron conditions to induce the high-affinity iron transport system. The *gef1–1* allele was derived from parental strain JY102 and has been previously described (10). Cells were grown in yeast/peptone/dextrose (YPD; 1.0% yeast extract/2.0% peptone/2.0% glucose) or in YPD made iron-limited by the addition of bathophenanthrolinedisulfonate (14). DNA transformations of *Escherichia coli* and *S. cerevisiae* were performed by standard procedures (17, 18).

Iron Transport Assays. Cells were grown in either YPD or in YPD made iron limited by the addition of 80 μM bathophenanthrolinedisulfonate. The cells were washed and assayed for iron transport by incubation for 10 min at 30°C in Cl^--containing assay medium that contained 0.5 μM ^{59}Fe in the

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Abbreviations: PPD, *para*-phenylenediamine; CLC, chloride channel; CPY, carboxypeptidase Y.

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presence of 1.0 mM ascorbate (14). The assay medium was made without Cu, and where specified 0.25 μM $CuSO_4$ was added to the medium.

To assay for the Cu-loading of cell surface apoFet3p, cells (MM19) were incubated at 0°C for 30 min with 1 mM ascorbate and 2.0% glucose in "universal" buffer (14.3 mM citric acid/14.3 mM monopotassium phosphate/14.3 mM boric acid/14.3 mM barbital in which the pH was adjusted as specified with 0.2 M NaOH) in the presence or absence of $CuSO_4$ and NaCl. The cells were washed in Cu^+- and Cl^--free buffers, either universal buffer or citrate buffer, and iron transport was determined as described by Askwith *et al.* (14), except that the assay buffer was Cu^+- and Cl^--free 50 mM citrate (pH 4.5) containing 2.0% glucose and 1 mM ascorbate.

***para*-Phenylenediamine (PPD) Oxidase Activity.** Two modifications of the protocol of Yuan *et al.* (13) were used to examine Fet3p dependent oxidase activity. For the experiment shown in Fig. 1*B* cells were grown overnight in YPD medium made iron-limited by the addition of bathophenanthrolinedisulfonate. Membranes were isolated from cells, in the presence of 1.0 mM ascorbate with either the Cu chelator bathocuproinedisulfonic acid to prevent Cu-loading during cell lysis, or with 25 μM Cu^+ to facilitate Cu-loading of apoFet3p, the ascorbate ensuring the presence of Cu^+. Samples of membrane protein (30 μg) were loaded onto SDS/4–20% polyacrylamide gradient gels. The gel was run at 100 V, soaked in a Triton/glycerol solution (10.0% glycerol/0.05% Triton X-100), and then soaked in 100 mM sodium acetate (pH 5.7) in the presence of PPD·2HCl (Sigma).

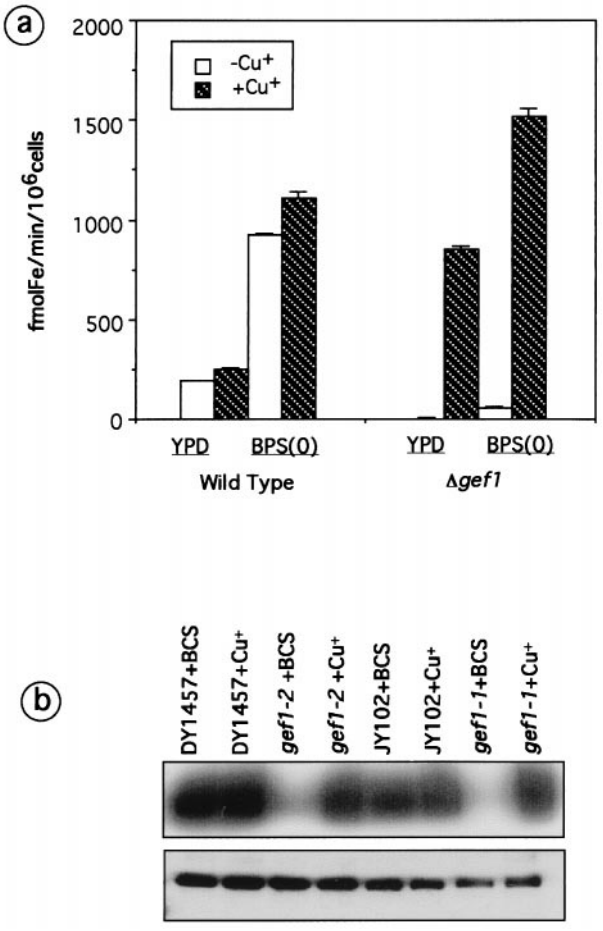


FIG. 1. (*A*) Copper dependence of iron transport in wild-type yeast and in Δ*gef1* cells. Wild-type (DY1457) and Δ*gef1* cells were grown in YPD overnight or in medium made iron limited by the addition of 80 μM bathophenanthrolinedisulfonate (BPS) for 4 h. (*B*) Fet3p-dependent PPD oxidase activity from wild-type and *gef1* cells. Membranes from wild-type and *gef1* cells were isolated either in the presence of the Cu chelator bathocuproinedisulfonic acid (BCS) or in the presence of additional Cu^+. PPD oxidase activity of the isolated membranes was determined as described in *Materials and Methods*. The *gef1–1* allele was derived from parental strain JY102, and the *gef1–2* allele was derived from parental strain DY1457. (*Upper*) PPD oxidase activity. (*Lower*) Western blot, to show equal loading of Fet3p.

A modification of the above procedure was employed for experiments that examined the Cl^- and pH dependency of Cu-loading of apoFet3p in isolated membranes. The membranes were washed in either universal buffer at pH 4.5 or pH 7.5, or in the more commonly used lysis buffer at pH 7.4. The membranes were resuspended in the universal buffer at the specified pH with 25 μM $CuSO_4$, in the presence or absence of 35 mM NaCl, or in lysis buffer (which contains 150 mM NaCl) in the presence or absence of 25 μM $CuSO_4$. All buffers contained 1 mM ascorbate. The membranes were incubated at 0°C for 30 min, and then washed twice by centrifugation using the appropriate buffer lacking Cu^+ and then once with lysis buffer. The pelleted membranes were incubated in 35 μl of lysis buffer containing 1.0% Triton X-100 and protease inhibitors at 0°C for 16 h. The extract was centrifuged at 16,000 $\times$ *g* for 30 min, and the supernatant was removed for measurement of PPD oxidase activity. Equivalent samples (15 μg) were also examined for Fet3p by Western blot analysis. Samples were applied to an SDS/4–20% polyacrylamide gel under standard conditions, using an antibody directed against the C terminus of Fet3p to visualize the protein (19). The blots were washed and probed with a horseradish peroxidase-conjugated goat anti-rabbit antiserum (Jackson ImmunoResearch) and developed by using the enhanced chemiluminescence detection system (Amersham).

Additional Methods. Secretion of carboxypeptidase Y (CPY) was determined by labeling cells with [^{35}S]methionine and examining both cells and media for the presence of CPY as described by Radisky *et al.* (20). Protein determinations were performed using the BCA (bicinchoninic acid) reagent (Pierce), using BSA as a standard.

RESULTS

Previously, we identified a *gef1* mutant that was unable to grow on low-iron medium (10). Using a different selection system and yeast strain, we recently identified a second *gef1* allele (*gef1–2*) unable to grow on low-iron medium. We measured high-affinity iron transport activity in the presence and absence of Cu^+ in strains carrying both *gef1* alleles as well as in a *gef1* deletion strain. As expected, wild-type cells grown in iron-replete medium showed low activity of the high-affinity iron transport system, because transcription of both *FET3* and *FTR1* is repressed in iron-replete medium (14, 21) (Fig. 1*A*). High-affinity iron transport activity was not observed in Δ*gef1* cells when the cells were assayed in the absence of Cu^+ (Fig. 1*A*). High-affinity iron transport in the *gef1–1* and *gef1–2* mutants was also found to depend on extracellular Cu^+ (data not shown). Relative to wild-type cells, abnormally high levels of transport were seen in the Δ*gef1* strain in the presence of added Cu^+. This result indicates that the high-affinity iron transport system was induced but inactive in the absence of added Cu^+. When wild-type cells are starved for iron there is an induction of the high-affinity iron transport system. Little iron transport activity was observed in the Δ*gef1* strain when Cu^+ was absent during the assay period, but the addition of Cu^+ leads to robust iron transport activity. These results indicate that the *gef1* mutants have a defective high-affinity iron transport system due to an inability to Cu-load apoFet3p.

This hypothesis is confirmed by measurement of the ability of Fet3p from wild-type and mutant cells to oxidize the substrate PPD. Fet3p, like other multicopper oxidases, can utilize PPD as a substrate. Although iron is the preferred

substrate, oxidation of PPD can be used as a measure of enzymatic activity. To date, any mutation or alteration that affects ferroxidase activity and iron transport similarly affects PPD oxidation. Extracts from control cells showed abundant PPD oxidase activity, but extracts from *gef1–1*, *gef1–2* cells showed little activity (Fig. 1*B*). Addition of Cu^+ to membrane extracts of wild-type cells resulted in a small increase in PPD oxidase activity, whereas Cu^+ addition to *gef1* extracts resulted in the appearance of PPD oxidase activity comparable to wild-type levels. Under normal conditions Fet3p is Cu-loaded within a subcellular compartment. Defects in the intracellular Cu-loading of Fet3p result in the appearance of apoFet3p on the cell surface. Similar results have recently been published by Gaxiola *et al.* (9).

A *gef1* defect might result in an unfavorable electrochemical potential difference that results from cation transport. Studies suggest that this potential difference may prohibit further transport of cations into vesicular compartments limiting either pH or Cu^+ concentration (7, 9). Mutations in the subunits of the vacuolar H^+-ATPase lead to an inability to grow on low-iron medium, which is suppressed by addition of Cu^+. This defect also results from defective Cu-loading of Fet3p (ref. 22 and data not shown), suggesting that an acidic pH may also be required for Cu-loading of apoFet3p. Yeast vacuolar pH mutants, however, show missorting of vacuolar proteins such as CPY (23). Many mutants in the secretory pathway (*vps* mutants) that missort CPY also have an enzymatically inactive Fet3p (20, 22). Examination of the biosynthesis of CPY did not show any significant missorting in *gef1–2*, and Δ*gef1* strains (data not shown). Thus, any effect of *gef1* on the pH of the compartment in which apoFet3p becomes Cu-loaded does not lead to missorting of secretory proteins. This result suggests that the Cu-defect does not result from defects in vesicle trafficking.

If the pH of the compartment in which apoFet3p obtains Cu^+ is normally acidic, then Cu-loading of apoFet3p might be pH dependent. To test this prediction, we devised an assay for the Cu-loading of apoFet3p. Cells with a deleted *CCC2* gene are unable to Cu-load apoFet3p, but they can appropriately synthesize and target apoFet3p and Ftr1p to the cell surface (13). Cell surface apoFet3p can be Cu-loaded at 0°C, in the absence of metabolic activity, by the addition of reduced Cu^+. The extent of the Cu^+-mediated reconstitution of Fet3p is assessed by uptake of ^{59}Fe at 30°C. The Cu-loading of apoFet3p at 0°C shows pH dependence, with the greatest activity at a pH of 4.5 (Fig. 2*A*). The pH dependency of Cu-loading of apoFet3p was independent of the pH at which iron transport activity was assayed. The pH of the mammalian trans-Golgi apparatus has been measured as 5.9–6.5 (24–26), but no measurements of the pH of the yeast trans-Golgi apparatus has been reported. An unexpected finding was that Cu-loading was markedly dependent on the presence of Cl^-. Cu-loading of apoFet3p at any pH was substantially less in the absence of Cl^- (Fig. 2*A*). Incubation of cells at 0°C or at different pH in the absence of Cl^- did not affect subsequent Cu-loading or iron transport activity (data not shown). This result demonstrates that neither low temperature nor low pH permanently affects the ability of apoFet3p to accept Cu^+. No iron transport was seen when Δ*fet3* cells were exposed to Cu^+ and Cl^-, showing that the observed iron transport requires Fet3p (data not shown).

At pH 4.5, the concentration of Cl^- that gave half-maximal loading at a Cu^+ concentration of 0.2 μM was 15 mM, with 75 mM Cl^- giving complete loading. These concentrations of Cl^- are well within the physiological range. Cu-loading of apoFet3p occurs at pH 7.4, although high concentrations of Cu^+ (25 μM) and Cl^- (100 mM) are required. apoFet3p was Cu-loaded when cells were incubated with NaCl or KCl, suggesting that the cation was irrelevant. A similar Cl^- dependence of apoFet3p Cu-loading was observed in citrate, acetate, or a complex buffer of citrate/borate/barbital/phosphate (universal buffer), indicating that the buffer was irrelevant. No Cu-loading was seen when cells were exposed to anions such as acetate, citrate, phosphate, or SO_4^{2-}, at concentrations as high as 100 mM. Other halides could also mediate the Cu-loading of apoFet3p: Br^- was as effective as Cl^- in loading Fet3p, whereas I^- was much less efficient. At a Cu^+ concentration of 0.3 μM, 35 mM I^- resulted in only 15%

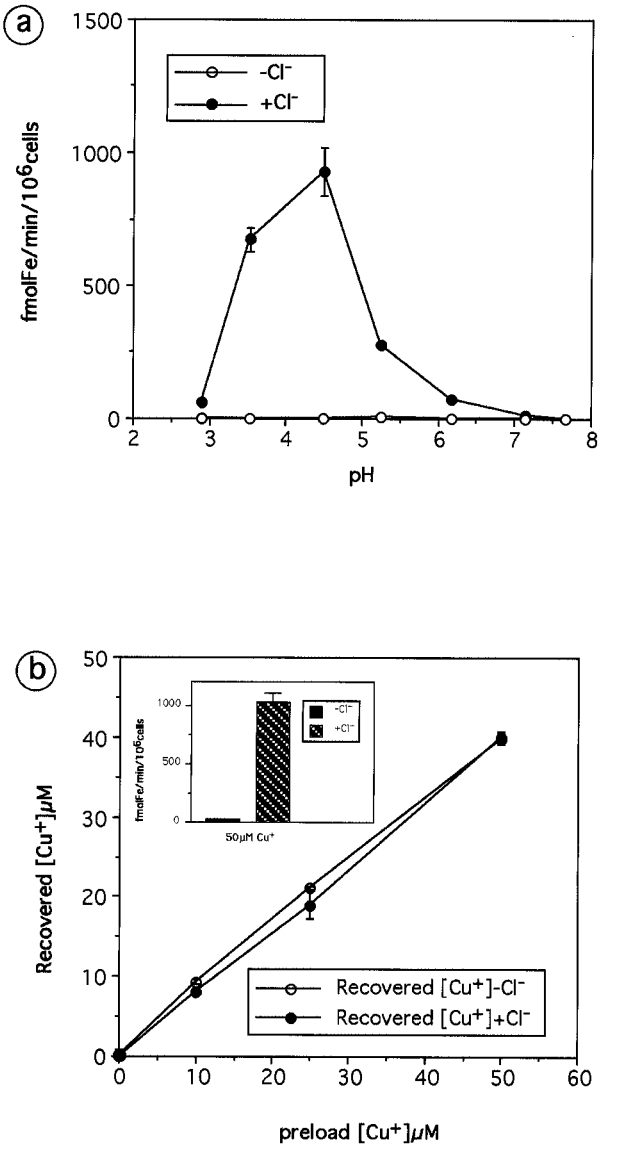


FIG. 2. (*A*) pH and Cl^- dependency of Cu^+ loading of apoFet3p. Yeast strain MM19 was grown in YPD. The cells were washed at 0°C with citrate buffer (pH 4.5) and then washed and incubated in universal buffer containing 2.0% glucose, 1.0 mM ascorbate, and 0.2 μM $CuSO_4$, in the presence and absence of 35 mM NaCl at the specified pH. After a 30-min incubation the cells were washed with citrate buffer and assayed for ^{59}Fe transport in citrate buffer containing glucose and ascorbate. (*B*) Effect of NaCl on the solubility of Cu^+. MM19 cells grown in YPD were washed in cold citrate buffer and resuspended to an OD_{600} of 28 in universal buffer (pH 4.5) containing 2% glucose, 1.0 mM ascorbate, and the specified concentrations of $CuSO_4$ in the presence or absence of 35 mM NaCl. The cells were incubated at 0°C for 30 min and pelleted by centrifugation, and the medium was removed. The cell pellet was resuspended, extensively washed with citrate buffer, and assayed for iron transport as described for *A*. The medium from the cell pellet was assayed for copper by using bathocuproinedisulfonic acid and measuring absorbance at 490 nm. (*Inset*) Rate of iron transport in cells exposed to 50 μM Cu^+ in the presence or absence of Cl^-.

of iron transport activity. Our results indicate, however, that once apoFet3p has been Cu-loaded, iron transport is independent of the presence of Cu^+ or Cl^- in the assay buffer.

When cells are exposed to Cu^+ there is a time-dependent absorption of Cu^+ to cell walls and membranes. Bound Cu^+ released at 30°C in the presence of ascorbate and Cl^- is capable of Cu-loading surface apoFet3p. Surface loading can be prevented by the addition of the Cu^+ chelator bathocuproinedisulfonic acid. We considered the possibility that Cl^- may function simply to maintain Cu^+ in a soluble form. We incubated cells with different concentrations of Cu^+ in the presence and absence of Cl^- at 0°C for 30 min, centrifuged cells, and then assayed the amount of Cu^+ in solution. The presence of NaCl had no affect on the amount of Cu^+ adsorbed to cells. At all concentrations tested >85% of Cu^+ remained in solution (Fig. 2*B*). In the absence of Cl^- even 50 μM Cu^+ was unable to load apoFet3p (Fig. 2*B Inset*), whereas in the presence of Cl^- as little as 0.2 μM Cu^+ gave rise to a fully active Fet3p (data not shown). Thus, the role of Cl^- is not simply to maintain Cu^+ in solution.

The effect of Cl^- on the Cu-loading of apoFet3p was also demonstrated *in vitro* by using isolated membranes as a source of apoFet3p. Exposure of membranes to Cu^+ in the presence of Cl^- resulted in the Cu-loading of Fet3p as assessed by the appearance of PPD oxidase activity (Fig. 3). Cu-loading of apoFet3p can occur at pH 7.4 with high Cl^- and high Cu^+. These conditions have been used previously to Cu-load both crude or pure apoFet3p (13, 27). There is a pH dependency of Cu-loading of apoFet3p when moderate concentrations of Cl^- are present (35 mM). In the absence of Cl^-, the addition of Cu^+ to apoFet3p does not lead to PPD oxidase at any pH. These results demonstrate that the effect of Cl^- on the Cu-loading of apoFet3p does not depend on the presence of intact cells and can be seen *in vitro*, independent of the assay method used.

Examination of the effect of Cl^- on the concentration dependency of Cu-loading reveals that Cl^- is a positive allosteric effector of Cu-loading, with a Hill coefficient for Cu^+ of 1.47 at a Cl^- concentration of 20 mM (Fig. 4*A*). The apparent Hill coefficient for Cu^+ varies with different concentrations of Cl^- (data not shown). The effect of Cl^- concentration on Cu-loading of apoFet3p is cooperative (Fig. 4*B*). At low concentrations of Cu^+ there is a sigmoidal effect of Cl^- concentration on the formation of active Fet3p. The sigmoidal effect is decreased at higher Cu^+ concentrations. Again, the apparent Hill coefficient for Cl^- is dependent on Cu^+ concentrations (1.4 at 10 μM Cu to 4.0 at 0.1 μM Cu). The variation in Hill coefficient precludes a simple kinetic interpretation of the data. It does suggest, however, that Cl^- may also bind to Fet3p, and that the interaction between Cl^- and Cu^+ is highly cooperative.

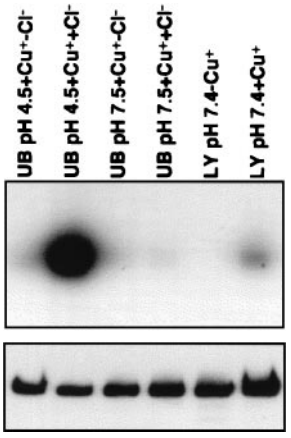
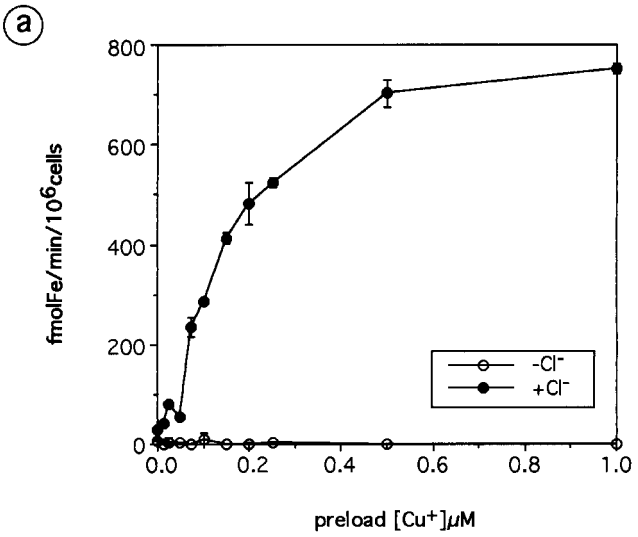


FIG. 3. Effect of Cl^- on the *in vitro* Cu-loading of apoFet3p. Isolated membranes were assayed for the Cl^- and Cu^+ dependency of Fet3p activity by using a modification of the PPD oxidase assay. The membranes were incubated in either universal buffer (UB) at the designated pH or lysis buffer (LY) at pH 7.4. The concentration of Cu^+ was 25 μM and the concentration of Cl^- in UB was 35 mM. (*Upper*) PPD oxidase activity. (*Lower*) Western blot demonstrating that equivalent amounts of Fet3p were present in all conditions.



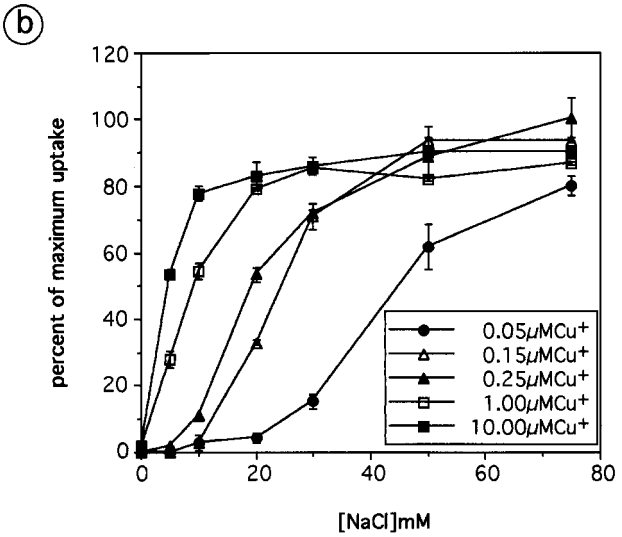


FIG. 4. (*A*) Effect of Cl^- on the concentration dependency of Cu-loading of surface apoFet3p. MM19 cells grown in YPD were washed in cold citrate buffer and incubated in universal buffer (pH 4.5) containing 2.0% glucose, 1.0 mM ascorbate, and the specified concentrations of $CuSO_4$ in the presence or absence of 20 mM NaCl. After a 30-min incubation the cells were washed and iron transport was measured. (*B*) Effect of Cu^+ on the concentration dependence of Cl^- in the activation of Fet3p. Cells were incubated with the specified amounts of Cu^+ and Cl^- at 0°C before assay of transport.

DISCUSSION

Our results indicate that Cl^- is an allosteric effector for the copper-loading of Fet3p. Cl^- has been reported to be required for the activity of ceruloplasmin, a mammalian multicopper oxidase involved in iron metabolism (28). Our results suggest that once apoFet3p has been exposed to Cl^- and reduced Cu the continued presence of Cl^- in the medium is not required for activity. This observation does not rule out an effect of Cl^-

on enzymatic activity independent of its effect on the metallation of the enzyme. An interesting possibility, suggested by the study of Musci *et al.* (28), is that Cl^- may bind to regions around the trinuclear cluster.

The mutual dependency of Fet3p activity on both Cu^+ and Cl^- may explain how disruption in Cl^- transport resulting from a defective *gef1* affects Fet3p activity. In addition to the generation of an unfavorable electrochemical potential, reductions in Cl^- transport may also affect enzyme activity, as Cl^- may be required for assembly of active metal-containing enzymes. Studies have shown a requirement of Cl^- for the *activity* of metalloenzymes, but we know of no other demonstration of a role for Cl^- in the *assembly* of Cu-containing proteins. Many secretory and resident vesicular enzymes require transition metals (Cu, Zn, Mn, and Fe) as cofactors. For example, Mn-glycosyl transferases exist in the Golgi (29), Zn-proteases reside on cell surfaces, Fe-acid phosphatases are present in lysosomes (30), and Cu-containing enzymes such as lysyl oxidase are secreted (31).

The mechanism of metal-loading of apoproteins within the vesicular apparatus is not well understood. Our studies suggest that specific anions may be required for the assembly of a number of metalloenzymes. Although little is known about the Cl^- requirement of vesicular enzymes, there is some intriguing information that may bear upon the pathophysiology of Dent's disorder. There is evidence that Cl^- is an allosteric effector for the metallation of the yeast vacuolar enzyme aminopeptidase I (32). Cl^- binding to aminopeptidase I is required for the assembly of the active Zn-containing enzyme, and Zn^{2+} is required for Cl^- binding. Aminopeptidase I may enter the vacuole through a nonclassical secretory route (33), so the site of Cl^- or Zn^{2+} addition is unclear. The fact that the enzyme requires Cl^- for activity suggests that the action of a vesicular or vacuolar Cl^- channel is required to obtain full catalytic activity. Additionally, presence of Cl^- has also been shown to increase the activity of arginine aminopeptidase, a metalloenzyme present in chromaffin granules (34). Thus, although there may be a number of vesicular enzymes that require Cl^-, either for metallation or for activity, at least two enzymes involved in protein hydrolysis require Cl^- for their activity. This requirement may be of particular relevance to Dent's disease, in which a major clinical finding is the excessive secretion of low molecular weight proteins. Possibly, defective protease activity resulting from ineffective Cl-dependent metallation may contribute to this clinical finding. Thus, a defective CLC might disrupt normal cellular function by causing defective cation homeostasis, and also by preventing metallation and suppressing the activity of many enzymes.

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