The Fet3 protein from *Saccharomyces cerevisiae* is a multicopper oxidase with specificity toward Fe(II) and Cu(I). Fet3p turnover of Fe(II) supports high affinity iron uptake across the yeast plasma membrane, whereas its turnover of Cu(I) contributes to copper resistance in yeast. The structure of Fet3p has been used to identify possible amino acid residues responsible for this protein’s reactivity with Cu(I), and structure-function analyses have confirmed this assignment. Fet3p Met^345_ is required for the enzyme’s reactivity toward Cu(I). Although the Fet3pM345A mutant exhibits wild type spectral and electrochemical behavior, the kinetic constants for Cu(I) turnover and for single-turnover electron transfer from Cu(I) to the enzyme are significantly reduced. The specificity constant with Cu(I) as substrate is reduced by one-fifth, whereas the electron transfer rate from Cu(I) is reduced 50-fold. This mutation has little effect on the reactivity toward Fe(II), indicating that Met^345_ contributes specifically to Fet3p reactivity with the cuprous ion. These kinetic defects render the Fet3pM345A unable to support wild type cellular copper resistance, suggesting that there is a finely tuned copper redox balance at the yeast plasma membrane.

Ferroxidases are a group of multicopper oxidases (MCOs) that exhibit an activity toward Fe(II) not shared by the other members of this family of copper proteins (1–4). MCO proteins are distinguished by having three distinct copper sites designated copper types 1, 2, and 3 (2, 4). All MCO proteins couple the oxidation of four molecules of a one-electron donor substrate with the reduction of one molecule of dioxygen to 2H_2O. Although the substrate specificity of these enzymes is quite broad, the specificity that has been characterized in a cohort of MCO proteins toward Fe(II) as reducing substrate results in the oxidation of four molecules of a one-electron donor substrate to dioxygen (1, 3). This reactivity is not restricted to this group of MCOs, it is exhibited also by ferritin in the process of iron core formation in this protein (5).

Human ceruloplasmin (hCp) (6–8) and the yeast plasma membrane protein, Fet3p (9–14), are the best characterized ferroxidases. Genetic studies in both mammals and yeast have established that the activity of these enzymes is essential to iron homeostasis in their respective organisms (3, 15). For example, in yeast, the action of Fet3p on Fe(II) is coupled to the transport of the Fe(III) product into the yeast cell (16, 17); Fet3p-deficient yeasts are therefore iron-deficient (9, 18). The ferroxidase activity of these copper enzymes explains the large body of literature that has reported on an apparent link between copper and iron metabolism in eukaryotes (15, 19).

In previous work, we examined the role(s) that Fet3p and hCp might have in managing the copper redox balance as well. We demonstrated that both ferroxidases exhibited a robust activity toward Cu(I) as substrate (20) and that in *Saccharomyces cerevisiae*, the activity that Fet3p had toward Cu(I) contributed to the overall resistance that yeast exhibited toward copper (21). This was the first report of cuprous oxidase activity attributable to a eukaryotic ferroxidase; this activity has been reported also in the copper resistance protein in *Escherichia coli*, CueO (22, 23). We have suggested that MCO proteins that exhibit reactivity toward lower valent metal ions be designated metallooxidases (4, 20).

The crystal structure of the MCO domain of Fet3p now provides the context for a directed protein engineering approach to delineate the structural elements in Fet3p that support the activity it has toward Cu(I) (14). Here we provide kinetic data indicating that a methionine residue at the T1 copper site in Fet3p is required for full activity toward Cu(I) in comparison with the activity Fet3p has toward Fe(II) as substrate. The data suggest that although the Fe(II) and Cu(I) binding sites in Fet3p kinetically overlap, the pathways for electron transfer from the two metal ions to the T1 Cu(II) differ. The kinetic defects exhibited *in vitro* by Fet3 proteins carrying an amino acid substitution at this residue position are reflected in an increased sensitivity to copper when this mutation is made in the form of the enzyme found in the yeast plasma membrane. Methionine thiether ligation of Cu(I) in Fet3p fits the pattern of similar ligation in other cuprous ion- trafficking proteins in pro- and eukaryotes.

**MATERIALS AND METHODS**

**Strains, Media, and Culture Conditions**—The strain used in the majority of the studies described was AJ505, which was derived from DEY1457 (MATa can1 his3 leu2 trp1 ura3 ade6) (24). The AJ505 genotype is MATa can1 his3 leu2 trp1 ura3 ade6 fet3::His ftr1::TRP1 aft1::AFT1-1upKAN (25). The AFT1-
1

Fet3p production and the apparent membrane-spanning domain found in the carboxy-terminal region that is included in residues 559–586.

This strain is MATa trp1-63 leu2-3,112 gcn4-101 his3-609 ura3-52 AFT1-1

Expression of Fet3p from pDY148 in a E. coli expression system for the purification of soluble Fet3p (11). This strain is MATa trp1-63 leu2-3,112 gcn4-101 his3-609 ura3-52 AFT1-1

Construction of Fet3p Mutants—Mutant Fet3 alleles were constructed directly in pDY148 (for secreted, soluble Fet3p) and in pDY133 (for the native membrane-associated Fet3p) by site-directed mutagenesis using the QuikChange kit from Stratagene. Briefly, complementary primers were used in PCR amplification of the appropriate vector to generate Fet3pM281A and M345A single mutants; two sequential rounds of mutagenesis yielded the Fet3pM281A/M345A double mutant. Mutated Fet3 sequences were confirmed by automated fluorescence sequencing on an ABI PRISM 377 instrument. Vectors expressing the mutant proteins were transformed into yeast strain M2* for soluble protein expression (11) and in strain AJS05 for in vivo analyses of protein localization at the plasma membrane and copper sensitivity (25).

Protein Characterization—Room temperature UV-visible absorption spectra were recorded using a Varian Cary 50 spectrophotometer. Protein concentration was determined using the standard dye-binding Bradford assay using bovine serum albumin as the protein standard (27). Reduction potentials of the T1 copper were obtained by the poised potential method (12). The stopped-flow kinetic experiments were performed at 4 °C in 100 mM phosphate buffer, pH 6.5, with a final protein concentration of ~50 μM; reduction of the four Cu(II) atoms in Fet3p was followed by the decrease in T1 Cu(I) absorbance at 608 nm. The tubing, plungers, and valves were rendered anaerobic by washing with dithionite solution followed by several washes with degassed water or buffer; the system was operated under a positive pressure of N2 to maintain anaerobiosis. A minimum of three reduction-absorbance traces for a given reaction were subsequently fit to equations describing mono- or biphasic first-order reactions using the Prism 4 software.

Analysis of Copper Resistance—Strain AJS05, producing episomally encoded wild type or mutant Fet3 proteins along with wild type Ftr1-GFP was used for these experiments; the medium was synthetic complete medium without or with CuSO4 (1 mM). Stock cell suspensions in water of A600 nm = 1.0 (~2 × 107 cells/ml) were used to prepare three serial dilutions. Cell dilutions (4 μl) were spotted on the solid medium. All plates were incubated at 30 °C for 2–3 days and photographed.

Confocal Fluorescent Microscopy—The trafficking of Fet3p mutants to the yeast plasma membrane was confirmed by co-expressing Ftr1-GFP in strain AJS05 as above; correct localization of this fusion requires the correct localization of the Fet3p partner species (25, 29). Yeast cells (1 ml) were grown as described, pelleted, washed once with phosphate-buffered saline, and resuspended in 100 μl of the same buffer. Confocal images were obtained using a Bio-Rad MRC 1024 confocal system equipped with a 15-milliwatt krypton/argon laser and operating on a Nikon Optiphot upright microscope with an oil immersion ×60 1.4 numerical aperture objective. Optical sections were acquired at 0.5 μm, and the xy resolution was set at 0.2 μm using the instrument’s Lasersharp version 3.0 software. The images were processed using Confocal Assistant version 4.02.

RESULTS

Locating Potential Cu(I) Ligands in the Metallooxidase Site in Fet3p—The T1 copper, ferroxidase site in Fet3p is illustrated in Fig. 1. Kinetic analysis has shown that Fe(II) and Cu(I) act as substrates at overlapping sites on Fet3p (i.e. the productive binding of the two reductants to the enzyme is mutually exclusive) (20). Consequently, we examined the Fet3p structure for
possible Cu(I) ligands in the vicinity of the three residues that support the ferrous iron reactivity of Fet3p, Glu^{185}, Asp^{283}, and Asp^{409} (13, 30). Specifically, we looked for methionine residues, since Cu(I) prefers soft ligands like sulfur. For example, CueO has a copper-binding site that is methionine-rich (23). Two such possible copper ligands were located near the T1 copper, Met^{281} and Met^{345}. These are shown in Fig. 1, with the distances given between the T1 copper and the SD atom of each side chain. These two residues were targeted for mutagenesis.

**Physicochemical Characterization of Fetp Mutant Proteins**—
Fet3pM281A, FetpM345A, and the double mutant Fet3pM281A/M345A were produced and purified as described for Fet3p wild type and other mutant forms (11, 13, 31). The proteins purified with their full complement of four copper atoms (Table 1). This result was consistent with the wild type absorbance at both 330 and 608 nm that the mutant proteins exhibited (Fig. 2 and Table 1); the transition at 330 nm is due to the protein’s type 3, binuclear Cu(II) cluster, whereas the transition at 608 nm is due to the favorable charge transfer from CysS → Cu(II) at the T1 copper (Cys^{484} in Fig. 1). The X-band, cwEPR spectra of the mutant proteins were also wild type (i.e. the spin Hamiltonian parameters for both the T1 and T2 Cu(II) in the mutants were unchanged) (data not shown).

The T1 copper sites in the three mutants also had reduction potentials that were identical to wild type Fet3p. These potentials were determined by the poised potential method, whereby solution potential was buffered by the ratio of ferri- to ferrocyanide and the ratio T1 Cu(I)/T1 Cu(II) in Fet3p was determined by the absorbance at 608 nm. This latter ratio was then plotted versus the solution potential according to the Nernst equation to yield the midpoint reduction potential of the T1 copper.

These plots are shown in Fig. 3 with the corresponding $E^\circ$ values given in Table 1; the slopes of the lines fell in the range 64–52 mV. The absorbance, EPR, and potential data indicate that mutation of either or both of these methionine residues had little if any effect on the electronic structure of the T1 copper or upon its inherent (thermodynamic) reactivity as an acceptor (oxidant) in an electron transfer process.

**Steady-state Kinetic Characterization of Fet3p Mutant Proteins**—This reactivity was determined in two ways. First, the kinetic constants for steady-state turnover with either Cu(I) or Fe(II) as substrate were determined by quantifying O$_2$ uptake using an oxygen electrode. The data obtained in this analysis are presented in terms of molecular velocities ($v_{measured}/[Fet3p]$) in Fig. 4, A and B, respectively. The kinetic constants corresponding to the nonlinear fits of the primary data to the Michaelis-Menten equation are given in Table 2. The data for wild type Fet3p show that Fet3p has a more robust activity toward Fe(II) in comparison with Cu(I); the difference is reflected primarily in $K_m$ values and in the resulting specificity constants, or $k_{cat}/K_m$ values. These latter values for Cu(I) and Fe(II) are 2.1 and 9.4 $\mu$M$^{-1}$ min$^{-1}$, respectively, indicating that Fe(II) is...
The data were fit to the Nernst equation to generate the theoretical lines shown for Fet3p WT (A) and mutants M281A (Δ), M345A (▼), and M281A/M345A (●). The fitted midpoint potentials are given in Table 1; the slopes of the lines varied between 64 ± 4 (WT) and 52 ± 4 mV (M281A/M345A).

The kinetic analysis of single-turnover, T1 Cu(II) reduction in Fet3p indicated that Asp409 plays a more significant role in Cu(I) turnover than Glu185.

The kinetic behavior of Fet3pM345A with Cu(I) and Fe(II) as substrates was compared with the differential reactivity that these two substrates exhibited with the Fet3pE185A and D409A mutants. Both carboxylate side chains play multiple roles in defining the reactivity that Fet3p has with Fe(II) (13, 14, 28, 30). We tested whether these residues were specificity elements solely for Fe(II) oxidation as Met345 appeared to be for Cu(I) turnover. The kinetic constants for these two mutants with both substrates are given in Table 2 (last two lines). The 2-fold decrease in $k_{cat}$ exhibited by Fet3pE185A was seen with both substrates; in contrast, the ~3-fold increase in $K_m$ for Cu(I) as substrate was observed only in the D409A mutant, whereas Fet3pE185A was wild type in this respect. These data indicate that Asp409 plays a more significant role in Cu(I) turnover than Glu185.

**Cuprous Oxidase Activity of Fet3p**

FIGURE 5. Reduction of the Fet3p T1 Cu(II) site by Cu(I). A, the reduction of Fet3p WT and M281A, M345A, M281A/M345A, and D409A mutants, as indicated by 400 μM Cu(I), was followed by absorbance at 608 nm. The protein concentration in each reaction was 50 μM; the temperature was 25 °C. Kinetic and equilibrium experiments were run under strictly anaerobic conditions.

In Fet3pM345A, the $K_m$ for Cu(I) increased nearly 2-fold, whereas $k_{cat}$ was reduced by a factor of 2.5; the latter value in the double mutant was reduced even further. A similar decrease in the $k_{cat}$ for Fe(II) turnover was seen in the double mutant; nonetheless, with respect to the specificity constants for this mutant, Fe(II) is now favored by a factor of 26 (3.6 for Fe(II) versus 0.14 for Cu(I)). We conclude that Met345 contributes specifically to the cuprous oxidase reactivity exhibited by Fet3p.
arate equilibrium experiment, we demonstrated that fully oxidized wild type Fet3p (four Cu(II)) was completely reduced by as little as 4 electron equivalents of Cu(I); these data are shown in Fig. 5B and show that complete reduction of the enzyme was occurring in the stopped-flow experiment.

The single-turnover results paralleled the steady-state ones in that Fet3pM345A (and the double mutant) exhibited a marked reduction in intermolecular electron transfer from Cu(I) that was not seen in Fet3pM281A. We conclude that of the two residues targeted here, only Met345 is required for the cuprous oxidase activity in Fet3p. The 50-fold reduction in $k_{ET}$ for the Fet3pM345A or the double mutant indicates that Met345 contributes to electron transfer by providing a binding site for Cu(I) (indicated also by the steady-state $K_m$ data) and to the pathway by which the electron on the donor Cu(I) is coupled to the T1 Cu(II) acceptor.

The carboxylic groups of Glu185 and Asp409 in Fet3p provide the route for electron transfer from Fe(II) to the T1 Cu(I) (13, 30). Asp409 in Fet3p is conformationally homologous to Asp439 in CueO, a residue whose carboxylate side chain is thought to couple substrate Cu(I) to the T1 copper in that MCO (23). The T1 copper reduction kinetics of Fet3p(D409A) were examined to determine if the Asp409 carboxylate participated in electron transfer in Cu(I) oxidation by Fet3p. The data in Fig. 5A and in Table 3 indicate that the reactivity with Cu(I) as reductant is suppressed by an alanine substitution at Asp409; this reduction in electron transfer rate is probably due in part to the increase in $K_m$ for Cu(I) quantified for this mutant (Table 2, last line). Note that ET into the T1 Cu(II) of MCO proteins commonly exhibits biphasic behavior with the fast phase always the dominant kinetic pathway (7, 12, 13, 30). The molecular basis for this behavior has not been elucidated.

**Trafficking and Physiologic Activity of Fet3p Mutants, Including Copper Sensitivity**—We evaluated the physiologic significance of the protein engineering results by constructing a parallel set of methionine mutants in the membrane-bound, native form of Fet3p and producing these mutant proteins in a fet3Δftr1Δ-containing strain, AJ505, along with the requisite partner permease in the form of Ftr1-GFP. Using the latter protein’s localization to the yeast plasma membrane as a measure of the trafficking competence of a given Fet3p mutant (20, 25, 28), we examined the copper sensitivity of strains producing these mutant Fet3 proteins.

All of the methionine-containing mutant proteins were trafficking-competent, as shown by confocal fluorescence microscopy (Fig. 6). The negative control (second panel) illustrates the perinuclear and endoplasmic reticulum retention of Ftr1-GFP produced in the absence of any Fet3 protein. Note that the patchy fluorescence contiguous with the cell surface seen in this transformant is from the endoplasmic reticulum (and the Fet3p, Ftr1-GFP) that accumulates at the cytoplasmic face of the PM in yeast and other eukaryotes (25). Also included in this screen was the Fet3p(T1D) protein that lacks any oxidase activity due to the absence of a type 1 copper atom. As the image in the third panel shows, this inactive ferroxidase is fully competent in assembling in the yeast PM with Ftr1-GFP.

The Fet3(T1D) protein was included in a copper sensitivity assay as a control for the lack of this activity in the yeast PM despite the presence there of a Fet3p-Ftr1p protein complex. The sensitivity assay is illustrated by the growth plates shown in Fig. 7. Yeast producing wild type Fet3p in the PM was copper-resistant, whereas cells that lack Fet3p activity at all (the negative control, second column) or have the inactive Fet3pT1D failed to grow at 1 mm copper salt (third column). The pattern of sensitivity for the methionine mutants corresponded to the deficit that the mutants exhibited in the in vitro cuprous oxidase assays; cells producing the Fet3pM281A mutant had wild type copper resistance, whereas cells producing an M345A-containing Fet3 protein had a resistance only slightly greater than the negative control or cells producing the oxidase-negative Fet3p(T1D). The data indicate that the 4–12-fold decrease in $k_{cat}/K_m$ for the Met $\rightarrow$ Ala mutants with Cu(I) as substrate is sufficient to render the modified Fet3p ineffective in suppressing the cytotoxicity of extracellular copper. Note that the copper sensitivity due to the lack of enzymatically active Fet3p in the PM is not related to an iron deficiency in the host strain; it is due solely to the Cu(I) generated at the PM by the metalloreductase, Fre1p (21).

### TABLE 3

**Single-turnover T1 Cu(II) reduction rates with Cu(I)**

Kinetic data as shown in Fig. 6A was fit to a two-phase exponential function by a nonlinear regression analysis, providing the rate constants and S.E. values given in the table.

| Fet3p species | T1 Cu(II) reduction | Fast phase $k_{ET}$ | Slow phase $k_{ET}$ |
|---------------|---------------------|---------------------|---------------------|
| WT            | 11.1 ± 0.57         | 0.59 ± 0.03         |
| M281A         | 16.9 ± 0.62         | 0.46 ± 0.02         |
| M345A         | 0.21 ± 0.02         | 0.02 ± 0.00         |
| M281A/M345A   | 0.25 ± 0.01         | 0.01 ± 0.00         |
| D409A         | 1.88 ± 0.02         | 0.44 ± 0.02         |

**FIGURE 6. Plasma membrane localization of Fet3 mutant proteins.** The co-transformants examined for copper sensitivity in Fig. 7 were examined by confocal fluorescence microscopy to demonstrate that the Fet3p methionine mutants were competent to traffic to the yeast plasma membrane. Ftr1-GFP was used as the marker for this trafficking. Note that the localization of the permease partner to the PM was unaffected by any of the Fet3p substitutions examined (see negative control for example of mislocalization).
Cuprous Oxidase Activity of Fet3p

The neuroprotective function of ceruloplasmin is widely acknowledged and is most commonly discussed in terms of the well-characterized ferrous iron specificity of hCp (15, 32–34). The ferroxidase activity of the soluble hCp secreted by astrocytes (35) or the GPI-anchored hCp produced by neurons (36) is thought to protect the cells by kinetically preventing the accumulation of Fe(II); ferrous iron could participate in Fenton chemistry in which the hydroxyl radical (HO') is produced from the reaction of Fe(II) with hydrogen peroxide (37, 38). HO' is produced by the reaction of Fe(II) with O2 also (39). However, these reactions are not limited to ferrous iron; cuprous copper, Cu(I), is equally reactive (39, 40). Indeed, in some in vitro model systems, copper and not iron acts as cofactor in reactive oxygen species-dependent cytotoxicity (41). To the extent that copper contributes to cytotoxicity in vivo, there is obvious selective advantage of a defense mechanism with activity toward both low valent, redox-active metal ion species. Data in both prokaryotes and eukaryotes indicate that MCO proteins with metallooxidase activity very likely do protect against both iron and copper (20, 42, 43).

The specificity an MCO has toward the one-electron donor (reducing) substrate is determined by the relative rate of electron transfer from a given substrate to the T1 Cu(II), which is the acceptor site in this outer sphere ET process (2, 4, 44). An outer sphere electron transfer reaction rate is analytically given by the Marcus equation, Equation 1 (45).

\[ k_{ET} = \frac{4\pi}{h}\left(\frac{h}{2\pi k_B T}\right)^{1/2} \frac{S_{DA}}{K_m} \exp\left(-\frac{\Delta G^*}{k_BT}\right) \]  

(Eq. 1)

In the Marcus equation, the rate of electron transfer, \( k_{ET} \), is governed first by the steric term, \( S \), and the equilibrium constant for formation of the interaction complex between the electron donor and acceptor, \( K_m \). The steric term represents that fraction of the complex that is competent for electron transfer, reflecting what the enzyme kineticist would refer to as productive binding; thus, the product, \( SK_m \), is equivalent to \( 1/K_m \), since \( K_m \) is the effective substrate dissociation constant from a productive Michaelis complex. The electronic matrix coupling element, \( H_{DA} \), provides a measure of the relative conductivity of the combination of through-bond and through-space jumps the electron must navigate in its path from donor to acceptor molecular orbital. The thermodynamic driving force for this electron transfer is given by \( \Delta G^* \), related to the difference in reduction potentials of donor and acceptor by the Nernst equation. Last, the reorganization energy, \( \lambda \), is the energy released or absorbed by the T1 copper coordination site upon change in redox state, from Cu(II) to Cu(I). The absorption spectra, redox potentials, and EPR spectra indicate that the T1 copper site is not strongly perturbed upon mutation of either Met281 or Met345. Therefore, the reorganization energy is probably unchanged in these mutants relative to wild type.

The Marcus equation provides a checklist for delineating the origins of substrate specificity in an MCO. The kinetic data presented in Table 2 show that Fet3p presents a low valent metal ion binding site that favors the productive binding of Fe(II) in comparison with Cu(I) based on the respective \( K_m \) values; the kinetic constants for Fe(II) and Cu(I) turnover by hCp suggest a similar selectivity for Fe(II) in that protein (20). The M345A substitution reinforces this selectivity for Fe(II), because it decreases the productive (kinetic) binding of Cu(I). We infer that Met281 contributes to the Cu(I) but not the Fe(II) coordination site and specifically to the \( SK_m \) term for electron transfer from Cu(I).

However, the observed ~2-fold increase in \( K_m \) (or decrease in \( 1/K_m \)) can maximally reduce \( k_{ET} \) in the Fet3pM345A mutant by the same 2-fold amount in comparison with wild type. The single-turnover, T1 Cu(II) reduction rate in this mutant is reduced 50-fold, however. Therefore, Met345 contributes to additional factors in the Marcus equation; since the reorganization energy does not appear to change upon substitution at Met345, we infer that this residue participates in setting the driving force for electron transfer from Cu(I) and/or contributes to the conducting pathway that couples the donor and acceptor in this ET reaction. These putative contributions would account for the 25-fold decrease in rate not due to the change in productive binding alone.

The conducting pathway is described by \( H_{DA} \); \( H_{DA} \), in turn, is inversely proportional to the attenuation of the electron transfer probability due to the resistance in the electronic coupling framework (46). In this framework, covalent bonds are the most conductive, hydrogen bonds are slightly less so, and through-space jumps are least conductive, with their resistance to ET increasing as a function of the distance of the jump. In Marcus theory (Equation 1), \( k_{ET} \) varies as the square of the coupling element, so it is strongly dependent on this value. The two ET pathways from bound Fe(II) to the T1 Cu(II) in Fet3p are through Glu185–His489 and Asp409–His413 (14, 30). These were indicated in Fig. 1.

An Fet3p structure with a bound metal ion has not been reported. However, one has been reported for the CueO protein (23). CueO is an MCO with activity toward both Fe(II) and Cu(I) whose activity in general is enhanced by the presence of copper (22, 42, 43). The coordination sphere of this activating copper includes two Met SD atoms and the OD1 atoms from two Asp side chains. The OD2 atom in one of these Asp side

**FIGURE 7. Copper sensitivity of yeast expressing wild type and Fet3p mutants.** Yeast strain AJS05 (fet3Δ/Δ) was co-transformed with vectors encoding Ftr1-GFP and Fet3p WT or Fet3p mutant T1D, M281A, M345A, or M281A/M345A; the negative control was AJS05 transformed with the parental vector, pRS416. Cells were spotted on medium lacking leucine and uracil for plasmid selection in the absence or presence of 1 mM CuSO4. The image was taken after 2 days of growth at 30 °C.
chains (Asp$^{439}$) is hydrogen-bonded to the NE2 of His$^{443}$, one of the ligands to the T1 copper (23). This latter hydrogen bonding network is conserved in the T1 copper, Fe(II) binding site in Fet3p (Fig. 1) (14, 30). Our kinetic data indicate that Met$^{485}$ is part of the metallooxidase site in Fet3p, too, whereas the structure of Fet3p indicates that Cu(I) could bind between the SD of Met$^{485}$ and the OD1 atom of Asp$^{409}$ much as the activating copper binds in CueO. In this model, Asp$^{409}$ but not Glu$^{185}$ contributes to the ET pathway from Cu(I). The data for Fet3pD409A are consistent with this model in that this mutant and not Fet3pE185A exhibits deficits in both Cu(I) binding and electron transfer rate.

The cuprous oxidase activity of Fet3pM345A is reduced in comparison with wild type, but the mutant is certainly not inactive with a $k_{cat}/K_m$ value with Cu(I) as substrate that is one-fifth the value for Fet3p (Table 2). Nonetheless, this deficit in reactivity toward Cu(I) was sufficient to render a strain producing this mutant Fet3p in the PM unable to mount effective resistance toward copper in the growth medium. This result underscores the fine balance between the benefit and cost of an essential nutrient that is cytotoxic if not managed properly. Cuprous ion, which is generated at the yeast plasma membrane by the action of the metallooxidases Fre1p and Fre2p, is the substrate for the copper transporter, Ctr1p (47). The efficiency of Fet3p as a cuprous oxidase must be sufficient to prevent the accumulation of Cu(I) at the PM but not so robust as to siphon off Cu(I) from the copper uptake pathway. The data presented here indicate that the balance between cuprous ion uptake and cuprous ion-dependent Fenton chemistry is a fine one in as much as a relatively small -fold change in Fet3p cuprous oxidase activity shifts the balance toward the latter pathway. One suspects that a similar delicate redox balance is in play for copper and iron cytotoxicity in general.

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