Spectral and Kinetic Properties of the Fet3 Protein from *Saccharomyces cerevisiae*, a Multinuclear Copper Ferroxidase Enzyme*

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High affinity iron uptake in *Saccharomyces cerevisiae* requires Fet3p. Fet3p is proposed to facilitate iron uptake by catalyzing the oxidation of Fe(II) to Fe(III) by O$_2$; in this model, Fe(III) is the substrate for the iron permease, encoded by *FTR1*. Here, a recombinant Fet3p has been produced in yeast that, lacking the C-terminal membrane-spanning domain, is secreted directly into the growth medium. Solutions of this Fet3p at $>$1 mg/ml have the characteristic blue color of a type 1 Cu(II)-containing protein, consistent with the sequence homology that placed this protein in the class of multinuclear copper oxidases that includes ceruloplasmin. Fet3p has an intense absorption at 607 nm ($\epsilon = 5500 \text{ M}^{-1} \text{ cm}^{-1}$) due to this type 1 Cu(I) and a shoulder in the near UV at 330 nm ($\epsilon = 5000 \text{ M}^{-1} \text{ cm}^{-1}$) characteristic of a type 3 binuclear Cu(II) cluster. The EPR spectrum of this Fet3p showed the presence of one type 1 Cu(II) and one type 2 Cu(II) ($A_2 = 91$ and $190 \times 10^{-4} \text{ cm}^{-1}$, respectively). Copper analysis showed this protein to have 3.85 g atom copper/mol, consistent with the presence of one each of the three types of Cu(II) sites found in multinuclear copper oxidases. N-terminal analysis demonstrated that cleavage of a signal peptide occurred after Ala-21 in the primary translation product. Mass spectral and carboxyhydride analysis of the protein following Endo H treatment indicated that the preparation was still 15% (w/w) $\alpha$-glycosidase H; PNGase F, peptide: ascorbic acid oxidase; MES, 4-morpholineethanesulfonic acid; Endo H, endoglycosidase H; PNGase F, peptide:N-glycosidase F; fAAS, flameless atomic absorption spectrophotometry; EPR, electron paramagnetic resonance; ES, electrospray; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis.

Ceruloplasmin (Cp) is a 132-kDa glycoprotein found in the mammalian plasma (1–3). It is a member of the class of enzymes known as multinuclear copper oxidases that includes laccase (Lac) and ascorbate oxidase (AO) in addition to Cp (4–6). These enzymes contain three distinct types of Cu(II) sites. These include a type 1 or “blue” Cu(II) that, due its strong Cys-S $\pi$ to Cu$^{2+}$ charge transfer adsorption at $\sim$600 nm ($\epsilon = 4–6,000 \text{ M}^{-1} \text{ cm}^{-1}$), imparts the characteristic blue color to these proteins; a type 2 Cu(II) that exhibits the weak d-d transition(s) typical of tetragonal Cu(II); and a type 3 binuclear Cu(II) pair that are strongly antiferromagnetically coupled and therefore EPR-silent (6, 7). As the crystal structures of human Cp (hCp) (2, 3) and AO (8, 9) show, these latter three Cu(II) atoms comprise a trinuclear cluster whose geometry conforms to a near isosceles triangle. These three enzymes catalyze the four-electron reduction of dioxygen using a variety of one electron substrates (e.g. dimethylphenylenediamine, o-dianisidine, dihydroxyacetic acid). Cp is apparently unique in this class in using Fe(II) as substrate, thus also catalyzing what is referred to as the ferroxidase reaction as shown in Reaction 1. Indeed, hCp is relatively specific for Fe(II) (10, 11).

$$4\text{Fe(II)} + \text{O}_2 + 4\text{H}^+ \rightarrow 4\text{Fe(III)} + 2\text{H}_2\text{O}$$

**REACTION 1**

The recently reported crystal structure of hCp in addition demonstrated a binding site for Fe(II)/Fe(III) on the protein adjacent to the type 1 Cu(II) (2, 3). This site is likely to be the site of electron transfer from Fe(II) to the protein at the type 1 Cu(II), i.e. the ferroxidase site. An adjoining site for Fe(III) has also been described in hCp; it is thought that the Fe(III) in this “holding” site is subsequently transferred to serum transferrin (3, 12). This coupling of the ferroxidase activity of hCp to its iron-binding sites suggests a molecular basis for the link between Cp and iron homeostasis in mammals, i.e. in the plasma, Cp catalyzes the oxidation of Fe(II) released from both erythrocytes and other cell types so that the resulting Fe(III) can be bound by transferrin, thus suppressing the level of “free” Fe(II) in the circulation (12, 13). The correlation between the absence of serum hCp (and its ferroxidase activity) and lipid peroxidation on the one hand (14) and cellular degeneration in the substantia nigra on the other (15, 16), is consistent with this suggestion. The prooxidant activity of Fe(II) in biologic systems is well documented (17).

Recently, a new potential member of this class of copper proteins has been described in the yeast *Saccharomyces cerevisiae*, the Fet3 protein (18–21). The *FET3* gene was cloned as a result of complementation of an iron uptake deficiency in this organism (18). It encodes a protein of 636 amino acid residues with a predicted mass of 72.4 kDa. Analysis of the encoded protein suggests that Fet3p has three important structural features: 1) a single transmembrane-spanning domain in...
cluded in residues 559–586 indicating that Fet3p is a type 1 membrane protein; 2) 13 potential N-glycosylation sites; and 3) most importantly, regions of strong homology to the multini-
clear copper oxidases in the regions of those proteins that are
directly involved in the binding of the catalytic Cu(II) pro-
thetic groups. Thus, alignment of the predicted Fet3p sequence
with the sequences of hCp, AO, and Lac shows that Fet3p very
likely has one each of the three types of copper sites found in
biology as are found in the other members of this class of
cuproproteins.

Fet3p is essential to iron uptake in yeast in two complemen-
tary ways. First, Fet3 protein is required for the correct tar-
geting of the putative iron permease, the Ftr1 protein, to
the plasma membrane. Deletion of the FET3 gene results in
the intracellular accumulation of Ftr1p (22). These and other
data indicate that Ftr1p and Fet3p comprise a protein complex
in the yeast plasma membrane. Second, active Fet3 protein
is required for iron uptake in that copper-deficient cells lack high
affinity iron uptake and this defect can be linked directly to
the inactivity of the Fet3p in the plasma membrane. Addition of
copper to these membranes restores the oxidase activity of
Fet3p that can be measured in vitro (20).

Fet3p does have ferrooxidase activity (21). Thus, although not
tested herein, the current model of high affinity iron uptake
is that the Fe(III) generated in the ferroxidase reaction
in Fe(II) oxidation to Fe(III) catalyzed by Fet3p is tightly linked
to translocation of the metal ion across the plasma membrane by the iron permease,
Ftr1p (20, 22, 23). That both Fet3p and hCp possess a ferroxi-
dase activity that is essential to their roles in iron homeostasis
in yeast and humans, respectively, suggests that delineation
of the yeast system, and, in particular, the structure-function
relationships in Fet3p, would contribute significantly to our
understanding of this apparent conservation of mechanism in
regards to iron trafficking in these two eukaryotes. Here, we
report the essential structural and kinetic features of a recom-
binant, soluble form of Fet3p that demonstrate unequivocally
that it is a multinuclear copper oxidase. Furthermore, using
appropriate steady-state kinetic approaches, we have obtained
a complete set of kinetic constants for the catalysis of the
ferroxidase reaction by this Fet3p protein.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Soluble Fet3 Protein—**Strain M2

—carrying plasmid pDY148 was used as the expression system for the
production of soluble Fet3p. Strain M2 is MATa trp1–190 leu2–3,112

gcn4–101 his3–609 ura3–52 AFT1–1 (24). The AFT1–1 allele en-
codes a dominant, gain of function mutant of the AFT1 protein, the
transactivator of the genes of the iron regulon, including
FET3 (25). Plasmid pDY148 is a 2μ vector that carries a recombinant FET3 effec-
tively truncated at nucleotide +1666 (at amino acid residue 555, below).

To construct pDY148, the wild type FET3 gene, including 13 potential

—glycosylation sites; 3) and,

—less atomic absorption spectrophotometry (fAAS) on a Perkin Elmer

—equipped with a model 700 graphite furnace. The protein was desalted on a Sep Pak C18 car-

—prior to injection. The mass peak was picked and assigned from the mass

—spectrometer. The protein was desalted on a Sep Pak C18 car-

—ions, was digested with trypsin (5ʿ of the transmembrane domain) and

—was digested with Endo H (as shown by SDS-PAGE) and the digested carbohydrate.

**Analytical Methods—Protein concentration was determined by two

—dye-binding one (Bradford) (29) and a rodox-based one (copper/biocinanchic acid) (30). Reducing sugar was
determined by the acid-anthene method (31). Standard Edman degra-
dation and carboxypeptidase methods were used for the N- and C-
terminal analyses, respectively. For the latter, seven times were
taken after amino acid analysis of the digestion mixture. Electrospray mass spectral analysis was performed on a VGFisons Qua-

—of the raw data of ion intensity versus m/z using an algorithm named “Max Entropy” (32). Copper was determined by flame-

—absorption spectrophotometry (FAAS) on a Perkin Elmer model 1100 instrument equipped with a model 700 graphite furnace.

**Spectral and Kinetic Measurements—**Absorbance spectra were rec-

—measured using the integrated form of the Michaelis-Menten equation, which allows for

—data to this equation was obtained by linear-least squares using the appropriate algorithm in the program

—software, Malvern, PA).
Fet3p, which appeared as a broad band, showed this material to be nearly homogeneous with respect to size-fractionation on a 7.5% acrylamide gel prior to staining with Coomassie Brilliant Blue R-250. The input sample in lane 1 was the eluate from the second monoQ column; the sample in lane 2 was from the third monoQ column used to remove the Endo H and free oligosaccharide (see “Materials and Methods”). Shown here also in lane 3 was material from lane 2 that had been subsequently size-fractionated on Superose12. No further purification was evident. Standard molecular size values are indicated to the left. The image was reproduced from a GelDoc file of the stained gel obtained from a Bio-Rad imaging system.

RESULTS

Expression and Purification of a Soluble, Secreted Form of Fet3—The native Fet3 protein contains a probable transmembrane domain that could consist of residues 559–686 (18, 22). This structural assignment was used to design a recombinant form of Fet3p truncated following residue Gly-555, with a tertiary FLAG epitope appended at that point for future immunologic detection and manipulation. That the resulting protein was secreted into the growth medium was consistent with this structure assignment. Furthermore, preliminary experiments demonstrated that this secreted protein exhibited an o-dianisidine oxidase activity that was dependent on the presence of a functional Ccc2 protein (23). Ccc2 is an apparent copper-dependent ATPase that is required for supplying copper for the activation of Fet3p within a compartment of the secretory pathway, most likely a late or post-Golgi one (20, 36). This Ccc2 dependence indicated that this soluble form of Fet3p was processed in an equivalent fashion to the native, membrane-bound protein.

The purification of this recombinant, soluble Fet3p from 10 liters of growth medium was accomplished in less than 2 days (see “Materials and Methods”). The adsorption of Fet3p from the growth medium by passage over a bed of monoQ was accomplished overnight. The protein was then eluted in batch by high salt, and the eluate diluted to lower the ionic strength and re-bound to a small monoQ column. It was again batch-eluted to concentrate the protein sample. SDS-PAGE analysis showed this material to be nearly homogenous with respect to Fet3p, which appeared as a broad band of ~120 kDa (Fig. 1, lane 1) as has been described for native Fet3p (20, 21). The heterogeneity of this material was undoubtedly due to a variable molecular carbohydrate content; Fet3p has 13 potential N-linked glycosylation sites (18, 20), and may also contain O-linked mannosae as well (below). This sample (~6 ml total volume from 10 liter of culture) was a blue crystal in appearance (Fig. 2) and exhibited a strong associated visible absorbance at 607 nm (Fig. 3). A shoulder at ~330 nm in the near UV portion of the spectrum was also apparent; this absorption was nearly equivalent in intensity to the one at 607 nm (Fig. 3).

Treatment of this sample with Endo H resulted in (some) trimming of the high mannosae core of the presumably N-linked carbohydrate to yield a species that migrated with an apparent mass of 85 kDa. This material appeared significantly more homogenous with respect to molecular mass based on the Coomassie-stained gel (Fig. 1, lane 2). This (partially, see below) deglycosylated protein exhibited essentially the same visible absorbance as did its precursor (data not shown); both preparations had an A278/A607 = 21.5 ± 0.5 (Fig. 3). For reference, this ratio for human ceruloplasmin is 22 (12). The Endo H-treated sample was then adsorbed to and eluted from a third monoQ column to remove the Endo H and released carbohydrate. The final preparation appeared >95% pure by SDS-PAGE (Fig. 1, lane 2). The yield from a 10-liter culture was ~40 mg of protein based on two independent protein assays (see “Materials and Methods”). Over 80% of the oxidase activity in the growth medium was recovered; the specific oxidase activity of this recombinant Fet3p was 3.0 μmol/min/mg of protein with o-dianisidine as substrate in a standard assay (28). A commercial preparation of porcine ceruloplasmin (Sigma) had a specific activity of 0.3 μmol/min/mg of protein.

N- and C-terminal Analyses of Recombinant, Secreted Fet3p—Based on the nucleotide sequence of the Fet3 gene, the predicted amino acid sequence for Fet3 protein includes an N-terminal hydrophobic leader motif that would be cleaved following targeting to the secretory pathway (18, 20, 36). In confirmation of this prediction, the N-terminal sequence of the soluble Fet3p was determined by Edman analysis to be ETHTF-. The yield at each cycle was nearly quantitative with respect to the amount of protein used, indicating the essential purity of the preparation insofar as polypeptide content was concerned. This N-terminal sequence indicates that the proteolytic processing occurs following Ala-21 in the primary translation product (18). The C-terminal sequence of this FLAG epitope-tagged recombinant protein was determined also to ensure that translation termination occurred appropriately at the engineered stop codons. Indeed, timed carboxypeptidase digestion followed by standard amino acid analysis showed that the C terminus was best represented by KD,KCO,H, where n = 3 or 4. The uncertainty in n arose from the presence of the DDDD motif in the FLAG epitope. Nonetheless, this result demonstrated that the Fet3 gene construct did terminate translation at the engineered stop codon(s). These protein sequence analyses indicated that the calculated protein molecular mass of this Fet3p, including the FLAG epitope, is 61,805 Da.

The compositional analysis of this material was also carried out. This was in agreement with the predicted amino acid composition (excluding those residues lost in the acid hydrolysis). Additionally, this analysis was in quantitative agreement with the solution protein assays, that is, the amount of protein (μg) recovered in this analysis was within 1% of the protein

2 D. S. Yuan, unpublished results.
amount indicated by the two assays used to evaluate the protein concentration prior to the amino acid analysis. This indicated that the standard dye-binding assay used to determine the Fet3p concentration in the spectroscopic and kinetic analyses described below gave an accurate measure of this quantity.

**Molecular Properties of Recombinant Fet3**—The molecular mass of this Endo H-treated, recombinant Fet3p was determined by electrospray mass spectrometry (ES/MS). The quality of the data as shown by the ion intensity versus mass transformation in Fig. 4 was not outstanding; despite rigorous sample preparation, the ion yield was low. One explanation of this was that the protein still had significant quantities of carbohydrate linked to it; carbohydrate can retard protein ion formation (37). Carbohydrate analysis confirmed this possibility (below). Nonetheless, a unique mass peak was observed at 72,872 Da. On the other hand, there were other peaks of both greater and smaller mass in the region ~70–76 kDa. The low ion yield, the indicated mass, and the apparent mass heterogeneity were all consistent with the likelihood that this preparation contained a significant and variable amount of carbohydrate linked to the population of Fet3p molecules. Thus, while the protein analyses above showed this preparation to be homogeneous with respect to amino acid content and polypeptide sequence, the MS analysis indicated that with respect to molecular species it was not. With respect to the major mass species of 72,872 Da, this value would indicate that this Fet3p preparation was still 15% (w/w) carbohydrate.

Subsequently, this protein preparation was analyzed also for copper content by flameless atomic absorption spectrophotometry (fAAS). The results of this analysis were compared with the protein concentration as determined above (see also “Materials and Methods”) and protein mass as determined by sequence analysis. Based on the protein concentration of the samples used for fAAS analysis and this molecular mass value, the stoichiometry of g atom copper/mol of Fet3p was calculated to be 3.85 ± 0.1. Using the molar concentration of Fet3p calculated in the same way, the molar extinction coefficient at 607 nm was determined to be 5500 M⁻¹ cm⁻¹, while that at 330 nm was calculated to be 5000 M⁻¹ cm⁻¹ (Fig. 3). The copper atom stoichiometry is consistent with the presumption that Fet3p is a classical multinuclear copper oxidase and is similar to that reported for the membrane-bound form of the protein (21). Most significantly, the intensity of the 607 nm transition demonstrated that Fet3p most likely contains the single type I Cu(II) site characteristic of these multinuclear copper proteins. It is the type 1 Cu(II) that contributes the intense blue color of the protein (Fig. 2) and its corresponding absorbance near 600 nm (Fig. 3). Furthermore, the transition at ~330 nm and its intensity are consistent with the presence of a type 3 binuclear Cu(II) site in Fet3p as well (6).

Carbohydrate analysis of this Endo H-treated preparation demonstrated that in treating the native protein with Endo H (or the related PNGase F, an amidase which cleaves the NAcGln-Asn linkage) considerable monosaccharide equivalents remained covalently linked to the protein. This analysis showed that this stoichiometry was 65 mol hexose/mol Fet3p (see “Materials and Methods”). The remaining carbohydrate may be O-linked through Ser/Thr; O-mannosylated proteins are common in *S. cerevisiae* (38). With an average mass per monosaccharide unit of 180 Da, this residual carbohydrate would contribute ~11.7 kDa to the total mass of this recombinant Fet3p (61.8 kDa), making the calculated mass of the partially deglycosylated Fet3p ~73.5 kDa. This value could be compared with the mass value assigned to the major peak in the ES/MS ion spectrum (72.9 kDa). Note, however, that none of these mass calculations takes into account the contribution of any other type of prosthetic group(s) linked to the Fet3p, e.g., copper, although in relative terms, this contribution would be small quantitatively.

**Electron Paramagnetic Resonance Spectral Analysis of Recombinant Fet3p**—Continuous wave EPR spectra of this Fet3p were obtained at 9.5 GHz (X-band) to characterize the nature of the four copper atoms present in the protein with respect to their oxidation states and likely ligand fields. These spectra clearly showed the presence of two separate Cu(II) sites with *g* values of 91 and 190 × 10⁻⁴ cm⁻¹, respectively (Fig. 5, hyperfine splitting in gauss). The *g* values associated with these two copper hyperfine interactions were 2.20 and 2.26, respectively. These *g* values and hyperfine couplings are consistent with the presence of both type 1 and type 2 Cu(II) in Fet3p. The value for *g*₁ was 2.05. The spectrum also exhibited the overshoot line common to a Cu(II) center that has a large *A*₁ value, e.g. a type 2 Cu(II) (39). The integrated intensities of each of these two spectral components were essentially identical; based on comparison to a Cu(II) standard and a molar concentration of Fet3p calculated as above, this integration was consistent with the presence of one type 1 and one type 2 Cu(II) per Fet3p. With respect to the *A*₁ = 91 × 10⁻⁴ cm⁻¹ resonance, this result is consistent also with the assignment of the *ε*₆₀₇ = 5500 M⁻¹ cm⁻¹ to a single type 1 Cu(II). In addition, the EPR spectra and their integration, together with the fAAS data, indicated that two copper atoms per Fet3p were apparently EPR-silent. This feature is consistent with but does not prove the presence of a strongly antiferromagnetically coupled type 3, binuclear Cu(II) cluster. However, the presence of the near UV absorbance expected for a type 3 site (Fig. 3; see above) is positive evidence for this assignment. In conclusion, the copper compositional and spectral data together strongly suggest that Fet3p is a multinuclear copper oxidase with one each of the three types of Cu(II) sites.

**Kinetic Analysis of the Ferroxidase Activity of Recombinant Fet3p**—Preliminary kinetic analysis of the ferroxidase activity of the membrane-bound form of Fet3p has been described, including estimated values for the *K*ₘ for Fe(II) (2 µM) and *V*ₘₐₓ (1.8 µM Fe(II) oxidized/min/10 µg of protein) (21). We wished to
extend these kinetic analyses to provide \( K_m \) values for both Fe(II) and O2, and a value for \( k_{\text{cat}} \) for both the glycosylated and (partially) deglycosylated forms of the protein. We expected this latter comparison to demonstrate any role the carbohydrate might play in the ferroxidase reaction itself, e.g. as a component of Fe(II) binding. To simplify the kinetic analysis, we took advantage of two facts: 1) the reaction is essentially irreversible, and 2) the products of the ferroxidase reaction, specifically Fe(III), are not inhibitors. These features allowed for the use of the integrated form of the Michaelis-Menten equation which, in one linear form, reduces to Equation 1 (34).

\[
2.3t\left(\frac{\log[S]_0}{[S]}\right) = \frac{-1}{[S]_0} + \frac{[S]_0}{V_{\text{max}}/k_{\text{cat}}} \quad \text{(Eq. 1)}
\]

Consequently, a plot of \([([S]_0 - [S]_t)/t] \) versus \( 2.3t/(\log[S]_0/[S]) \) yields values for \( K_m \) and \( V_{\text{max}} \).

Such data were obtained with respect to both [Fe(II)] and [O2], by simply following the time course of the reaction to completion with only one substrate limiting. Thus, with Fe(II) limiting, the consumption of Fe(II) was determined at [O2] \(_0\) \( \gg K_m \) by following the formation of Fe(III) spectrophotometrically at 315 nm (33). This time course was then analyzed at time intervals for the remaining [Fe(II)] and plotted according to Equation 1. This fit for Fe(II) oxidation catalyzed by the deglycosylated form of the Fet3p is shown in Fig. 6A; the fitted constants were: \( K_m \) [Fe(II)] = 4.8 \pm 0.3 \mu M and \( k_{\text{cat}} \) [Fe(II) turnover] = 9.5 min \(^{-1}\) (at [Fet3] = 0.2 \mu M). Velocity versus [O2] data were obtained from a trace of residual [O2] (determined by a standard O2 electrode and recorder with an expanded scale; see "Materials and Methods") in a reaction mixture initially containing 200 \mu M Fe(II) and up to 24 \mu M dissolved O2. Thus, in the latter condition, even after complete oxygen consumption and concomitant with the expected formation of 96 \mu M Fe(III), 100 \mu M Fe(II) would remain or over 20-fold greater than the \( K_m \) for Fe(II). These data are displayed in Fig. 6B as a fit to Equation 1 with respect to [O2] \(_0\). The fitted values were: \( K_m \) (O2) = 1.3 \pm 0.2 \mu M and \( k_{\text{cat}} \) [O2 turnover] = 2.3 min \(^{-1}\). The ratio of these two turnover numbers, 4.04, is consistent with the 4:1 Fe(II):O2 stoichiometry expected of the ferroxidase reaction. In addition, spectrophotometric analysis at 315 nm of the reaction mixtures used in the oxygen electrode experiments as a function of the initial [O2] showed that 3.9 \pm 0.2 mol of Fe(III) were produced for every mole of O2 consumed.
reaction catalyzed by soluble Fet3p.

Fe(II) oxidation (A) and O2 consumption (B) were measured continuously and the residual substrate concentration was plotted with respect to time according to the integrated form of the Michaelis-Menten equation as indicated in each panel. Fe(II) oxidation was followed by the appearance of Fe(III) at 315 nm while O2 consumption was determined by use of an O2 electrode (see "Materials and Methods"). The curve in each panel is a linear least squares fit of the data to Equation 1 (see "Results"). The [Fet3p] = 0.2 μM in 0.1 M MES buffer, pH 6.0, at 25 °C.

Similar experiments were carried out with the native, fully glycosylated form of the enzyme; the results were quantitatively equivalent with K_m values of 4.1 and 1.1 μM and k_cat values of 8.3 and 2.1 min^{-1}, for Fe(II) and O2, respectively, (data not shown). These results indicate that, at least with respect to the electron transfers from Fe(II) to dioxygen catalyzed by this soluble, truncated form of Fet3p, the (N-linked) carbohydrate moiety plays no apparent role in the ferroxidase reaction.

**DISCUSSION**

We have described the use of a yeast expression system that yields a soluble form of the yeast ferroxidase, Fet3p, in sufficient quantity for structural and mechanistic analysis. The importance of this is that Fet3p is catalytically essential to iron uptake by yeast and, furthermore, is a close homologue of ceruloplasmin, an enzyme strongly linked to and essential for normal iron homeostasis in humans and other mammals. Although the precise role that Cp plays in human iron metabolism is not known, there is abundant evidence that a deficiency in the level of circulating, ferroxidase-competent hCp at the least correlates with a variety of pathologies, predominantly ones that are associated with neurodegeneration. The clearest example of this correlation is given by the clinical manifestations of aceruloplasminemia that include diabetes mellitus, retinal pigment degeneration, and a complex of neurological symptoms (14, 15). Aceruloplasminemic patients also suffer from an increase in serum lipid peroxidation (14). The most probable link between this pathology, the lack of hCp, and the role of hCp in iron handling is that free iron, particularly Fe(II), is a strong prooxidant in biologic systems, *i.e.* the molecular basis of aceruloplasminemia could be due to a mishandling of circulating iron due to the absence of hCp and a consequent increase in iron-dependent oxidative damage (14, 16). Neuroimaging studies have revealed iron deposition in the basal ganglia of aceruloplasminemic patients (15).

The precise role of Fet3p in iron handling in the yeast *S. cerevisiae* is also unclear although, like hCp, the copper-dependent ferroxidase activity Fet3p is equally essential to normal iron trafficking in this microorganism. Furthermore, although how this Fet3p-dependent ferroxidase reaction is coupled to iron uptake is not known, minimally it most likely leads to Fe(III) binding to the Ftr1 protein, the putative iron permease (22, 23). Genetic and biochemical evidence strongly suggest that Fet3p and Ftr1p are closely associated in the yeast plasma membrane; consequently, the Fe(III) product of the ferroxidase reaction catalyzed by Fet3p would be in close proximity to the putative Fe(III) binding site(s) on Ftr1p. Indeed, the homology between mammalian and yeast iron handling is seen also in the presence of a series of glutamic acid-containing motifs in Ftr1p that are similar to elements in ferritin that are part of the channeling of Fe(III) from the ferroxidase center in that protein to the iron core (22, 41, 42). Although much of this model is still speculative, there is good reason to suggest that understanding the structural and functional relationships between Fet3p and Ftr1p will offer considerable insight into the coupling of ferroxidase and Fe(III) channeling activities in humans as well as in yeast. Detailed studies on Fet3p are critical to the elucidation of these relationships; the recombinant Fet3p described here clearly provides an excellent system for these studies.

**Protein Characterization**—The data presented show that this soluble, truncated form of Fet3p is a polypeptide of 61.8 kDa, including the FLAG epitope. In particular, the N-terminal analysis demonstrated the point of cleavage of the leader sequence that in the nascent polypeptide targets the protein to the endoplasmic reticulum; this cleavage occurs following residue 21, an alanine in the immature protein. The MS data indicated that following Endo H treatment, the resulting Fet3p appeared somewhat heterogeneous with respect to mass; comparison of the calculated and experimental mass values indicated that copper and the residual carbohydrate accounted for 11.1 kDa. In addition to 3.9 copper atoms per molecule indicated by the fAAS analysis, this residual mass would include the mass equivalent of ~65 hexose units per Fet3p molecule. Since the residual carbohydrate was resistant to hydrolysis by an enzyme with activity toward Asn-GlcNAc linkages (PNGase F) as well as to Endo H treatment suggested that the remaining hexose was associated with O-linked oligosaccharide as is common in yeast glycoproteins (38). The MS spectrum indicated also that the amount of this residual carbohydrate was somewhat variable in the population of Fet3p protein molecules in that the ion intensity data demonstrated the presence of minor mass peaks in addition to the major species of 73 kDa. Note that this mass heterogeneity was not due to protein heterogeneity since the amino acid analysis, and the N- and C-terminal analyses all showed this preparation to be homogeneous with respect to polypeptide composition. We do not know how the residual carbohydrate is linked to the Fet3p protein; no
Copper hyperfine interactions ($A_i$) at Cu(II) sites in multinuclear copper oxidases

| Protein                     | $A_i$ (type 1) | $A_i$ (type 2) | Ref. |
|-----------------------------|----------------|----------------|------|
| Laccase                     |                |                |      |
| *Rhus vernicifera*          | 39             | 182            | 45   |
| *Neospora crassa*           | 92             | 193            | 48   |
| Ascorbate oxidase (Cucurbita pepo medullosa) | 56     | 199            | 47   |
| Ceruloplasmin               |                |                |      |
| Human                       | 95             | 189            | 48   |
| *Cow*                       | 76             | 158            | 49   |
| Fet3p (*S. cerevisiae*)     | 91             | 190            | This work |

Histidine ligands and a water molecule or hydroxide. In the case of Fet3p, the nitrogenous ligands predicted by sequence alignments are His-81 and His-416. Of interest is the fact that, in both hCp and AO, the unoccupied type 2 Cu(II) site is oriented toward the center of the trinuclear cluster that includes also the pair of copper atoms that comprise the type 3 binuclear Cu(II) pair. Although these latter two Cu(II) atoms are each four co-coordinate, their coordination geometry has been described as trigonal bipyramidal with one empty equatorial site oriented also toward the center of the cluster. This electronic feature has been linked to a model of ligand binding to the cluster in which a ligand like azide bridges the type 2 with one of the type 3 copper atoms (9). In this model, the dioxygen binds to the cluster in this manner and is subsequently reduced by four electrons with the final kinetic step the expulsion of the second water oxygen from this bridging position (6, 50). Kinetic studies to test this model for this soluble form of Fet3p are in progress.

Type 3 Cu(II) was indicated in Fet3p most directly by the presence of a transition at ~330 nm with $\epsilon = 5000 \times 10^3$ cm$^{-1}$ that appeared as a shoulder on the absorption due to aromatic amino acid side chains in the protein. The energy and intensity of this transition is typical of type 3 Cu(II) in Lac and AO, for example (51). In the preparation of Fet3p as isolated this binuclear Cu(II) pair was apparently fully oxidized since addition of up to 30 equivalents of H$_2$O$_2$ had no effect on this absorption (data not shown). Reduced type 3 sites are readily oxidized by peroxide (51). The presence of a binuclear pair of Cu(II) was also inferred from the fact that two copper atoms per Fet3p molecule were EPR-silent at least down to 20 K. This result was consistent with the strong antiferromagnetic coupling of type 3 binuclear Cu(II).

Spectral Characterization—The presence of a type 1 Cu(II) site in this recombinant Fet3p was apparent immediately upon the batch purification of the growth medium on monoQ: a blue band was clearly adsorbed at the top of the resin. The resulting solution eluted with high salt was similarly deep blue (Fig. 2) and, as expected, exhibited an intense visible absorbance at 607 nm. The energy and intensity of this transition is a signature of type 1 Cu(II) (4–7). These sites are formally tetrahedral, although not necessarily strictly four-coordinate. At the least, however, all contain a single Cys ligand that contributes to the strong S to Cu(II) charge transfer transition represented by the ~600 nm absorbance. The type 1 site in Fet3p was predicted based on sequence homology to hCp and the other multinuclear copper oxidases. In Fet3p, this site likely includes His-413, Cys-484, and His-489 (the residue number is referenced to the mature protein). Unlike the type 1 sites in hCp (2, 3) and ascorbate oxidase (8, 9), however, which include a Met (via the mercaptide sulfur) in addition to two His and one Cys as ligands, the putative fourth coordination position in Fet3p would be vacant since sequence alignment places Leu-494 in this position. Leu is also in this position in the fourth member of this protein family, laccase (5, 6).

The X-band EPR data also indicated the presence of a type 1 Cu(II) in that a small nuclear hyperfine interaction in $g_z$ was clearly evident. A Cu(II) hyperfine coupling of this magnitude is typical of the strong S-Cu covalency characteristic of a type 1 Cu(II) site; this bonding leads to significant delocalization of unpaired electron density onto the cysteine ligand and thus away from the copper nucleus (7). Importantly, these spectra also provided strong evidence for the presence of the second of the paramagnetic Cu(II) sites found in multinuclear copper proteins, the type 2 Cu(II). A comparison of the A$_i$ parameters for these two Cu(II) sites in Fet3p to these parameters in the other multinuclear copper oxidases is given in Table I. The type 2 or “non-blue” Cu(II) site is approximated in ascorbate oxidase and in hCp by a T-shaped coordination geometry (2, 3) that, in the case of ascorbate oxidase, has been modeled as being electronically square planar with one empty equatorial coordination site (6). The three occupied sites at the type 2 Cu(II) in hCp (2, 3) and ascorbate oxidase (9) contain two
Spectral and Kinetic Properties of Fet3p

highly glycosylated and was of unknown mass, the precise molar concentration of Fet3p in those experiments could not be determined (21). As noted above, the \( V_{\text{max}} \) value was calculated with respect to mg protein, not a molar amount of Fet3p; a \( V_{\text{max}} \) was determined in this way to be 1.8 \( \mu \text{M}/10 \mu \text{g protein} \) (21). This latter value can be compared with the \( k_{\text{cat}} \) value determined here, 9.5 min\(^{-1}\), in the following way. The previous experiments were performed in a total volume of 200 \( \mu \text{l} \) (21); thus, assuming a protein mass for the Fet3 protein used in those experiments of 70 kDa, the [Fet3p] was \(-0.7 \mu \text{M}\). Thus, a very comparable turnover number of 2.6 min\(^{-1}\) can be estimated (1.8 \( \mu \text{M}/0.7 \mu \text{M}\)). Use here of a soluble Fet3p whose mass was precisely known allowed for the more precise calculation of \( k_{\text{cat}} \) values, which could then be compared with those obtained for the hCp-catalyzed ferroxidase reaction. With respect to the \( k_{\text{cat}} \) value for Fe(II) turnover, the value for Fet3p of 9.5 min\(^{-1}\) can be compared with that for hCp of 138 (12) or 550 min\(^{-1}\) (10) (depending on the method of analysis) or 10–50 times greater than for Fet3p.

The difference in the catalytic turnover by the two enzymes is not known. For the hCp reaction, the reoxidation half-reaction is rate-limiting; however, within that half-reaction, the rate-limiting step is \([O_2]\)-independent. This result was shown by Osaki and Walaas (11), who suggested that a slow protein conformational change precedes a fast, kinetically silent but \( O_2 \)-dependent reoxidation. This kinetic analysis was performed using the absorbance at 610 nm as a reporter of enzymic “reoxidation.” Of course, this absorbance reflects only the redox state of the type 1 copper site. Thus, an equally plausible interpretation of the Osaki and Walaas result is that the rate-limiting step in the hCp ferroxidase reaction is the electron transfer from the reduced type 1 copper to the trinuclear center with dioxygen bound presumably as a peroxy (two-electron reduced) intermediate as proposed for Lac (6, 50). In this model, the remaining electron in the cluster along with the electron transferred from the type 1 copper reduces the peroxy intermediate to the level of two water molecules concomitant with the “reoxidation” of the protein.

In the case of Lac, electron transfer from the type 1 Cu(I) is not rate-limiting; the rate-limiting step is substrate oxidation (54). This differs significantly from the ferroxidase reaction catalyzed by hCp in which substrate oxidation \([\text{of Fe(II)}]\) is \(10^2\)-fold greater than substrate oxidation \([\text{of dimethylphenylenediamine}]\) by Lac and in which enzyme-catalyzed reaction a step or steps subsequent to substrate oxidation is rate-limiting. In terms of the enzymic redox state, for Lac the reductive half-reaction is rate-limiting while for hCp the oxidative half-reaction is. This discussion of the enzymic half-reactions could be relevant to the fact that Fet3p turnover is 1/50 that of hCp. One explanation of this difference could be that the electron transfer from the type 1 copper, perhaps rate-limiting in the hCp reaction, is even slower in the case of Fet3p. Another possibility is that in the Fet3p reaction, as in the one catalyzed by Lac, the reductive half-reaction is rate-limiting, that is, the electron transfer from Fe(II) to the type 1 Cu(I).

One structural difference between hCp and Fet3p that could bear on the difference in turnover number in this regard is the presence of a coordinating Met in the catalytic type 1 Cu(I) site in hCp; in Fet3p, based on sequence alignment this residue position is occupied by Leu (6, 18). Sequence alignment places Leu at the type 1 site in Lac also (5, 6). The contribution of this ligand to the redox and electron transfer properties of the type 1 copper in the blue copper proteins has been most formally assessed in studies on wild type and mutant forms of azurin (55, 56) and on plastocyanin (57) and stellacyanin (58). These are all mononuclear type 1 copper proteins, which serve as electron mediators in bacterial electron transfer pathways. Although the Cu(II) sites in each of these proteins contain the basis set of ligands (His, His, and Cys), only azurin and plastocyanin contain also a Met (stellacyanin has a Gln), and, even in the former two proteins, the bonding of the Cu(II) to the methionine sulfur differs. These differences in coordination type and conformation at the least contribute to differences in mid-point potentials and electron exchange rates (55, 56).

The relevance of these comparisons to an understanding of the difference in ferroxidase activity of Fet3p in contrast to hCp is that the type 1 Cu(II) coordination site in hCp (and in AO) is the canonical one consisting of His, His, Cys, and Met. In this one regard, Fet3p is homologous to Lac since the type 1 sites in both proteins appear to be minimally three-coordinate since in both proteins the Met is replaced by Leu. Consistent with the increase in the midpoint potential of the type 1 Cu(II) in azurin due to replacement of Met-121 by a strictly hydrocarbon residue (55, 56), the potentials of the type 1 sites in the laccases range from 394 to 790 mV, all well above the reduction potential for any of the mononuclear type 1 copper proteins (6).

The redox potential of the type 1 Cu(II) in Fet3p has not yet been determined. Based on the discussion above, however, we would predict that it is more like that for the Lac type 1 site than for the catalytically active type 1 site in hCp. The redox potential for this site in hCp also is not known explicitly since hCp actually has two redox active type 1 copper atoms; available data indicate a range for these potentials between 370 and 580 mV (6) with the lower value more probably attributable to the type 1 copper in domain 6 of hCp that is linked structurally and presumably functionally to the trinuclear cluster which resides at the interface between domains 1 and 6 (2, 3). If this assignment is correct, it would suggest that the specificity that hCp and Fet3p exhibit toward Fe(II) as substrate in contrast to the other oxidases is not directly attributable to a particular midpoint potential. Indeed, the type 1 Cu(II) in any of these proteins, even one with an \( E^{\text{red}} \) = 370 mV, is oxidizing relative to Fe(II).

More likely to account for the activity toward Fe(II) demonstrated by hCp and Fet3p is some feature of the protein that leads to productive binding of this potential reductant. In the case of hCp, the crystal structure has provided some insight into this question. Analysis of the surface charge distribution has identified a strongly positive patch that is found in a valley created by three overhanging loops of polypeptide chain. One loop is contributed by each of the three domains in hCp (3). The type 1 Cu(II), the site of entry of electrons, is at the floor of this valley, indicating that the specificity toward Fe(II) may result from the combination of electrostatics and molecular sieving. Fet3p, however, will not likely conform exactly to this model since, based on sequence analysis, it does not obviously have the three-domain structure found in hCp and therefore would not be expected to have this exact surface topology (2, 3, 18). Indeed, the possible lack in Fet3p of elements found in the surface contour and charge distribution exhibited by hCp may underlie the diminished capacity that Fet3p has as a ferroxidase. It is also possible that association of Fet3p with the iron permease, Ftr1p, as occurs in the yeast plasma membrane, leads to an enhancement of this capacity in comparison to the \emph{in vitro} measurements described here. Such an interaction might also modulate the kinetic affinity that Fet3p exhibits toward Fe(II) thus accounting for the difference in \( K_m \) values for Fe(II) in \emph{in vitro} 59Fe uptake (0.15 \( \mu \text{M}\)) and in the \emph{in vitro} ferroxidase reaction (4 \( \mu \text{M}\)). Studies are under way to test these possibilities.

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