Spectroscopic Characterization of the Cu(II) Sites in the Fet3 Protein, the Multinuclear Copper Oxidase from Yeast Required for High-Affinity Iron Uptake

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Ceruloplasmin (Cp) is a member of the class of enzymes known as multinuclear copper oxidases[2] which also includes laccase[2] and ascorbate acid oxidase[3]). Ceruloplasmin exhibits a unique ferroxidase activity, that is, it and not the other known members of this class of copper proteins catalyzes the reaction shown in eq 1.[4]

$$4\mathrm{Fe(II)} + \mathrm{O_2} + 4\mathrm{H^+} \rightarrow 4\mathrm{Fe(III)} + 2\mathrm{H_2O} \qquad (1)$$

The ferroxidase activity of Cp is postulated to explain the link between copper and iron metabolism in mammals in that Cp catalyzes the conversion of ferrous to ferric iron in the plasma after the binding of the Fe(III) to transferrin.[5] Clearing free iron from the circulation by this ceruloplasmin-dependent mechanism appears essential to iron homeostasis in humans. This is indicated, for example, by the severe neural degeneration and increased lipid peroxidation which can be linked to the lack of circulating, active ceruloplasmin.[6]

A new member of this group of copper oxidases has recently been suggested by sequence homology and enzymic activity. This is the Fet3 protein from the yeast *Saccharomyces cerevisiae*.[7] DNA sequence analysis of the *FET3* gene strongly suggests that the encoded protein has one each of the three types of Cu(II) sites found in each of the members of this enzyme class: one type 1, or blue Cu(II); one type 2, or nonblue Cu(II); and one type 3 binuclear cluster of two strongly antiferromagnetically coupled and therefore EPR-silent Cu(II) atoms.[1,8] Sequence and biochemical analyses indicate that Fet3 is a type 1 transmembrane protein with a single, C-terminal membrane-spanning domain.[7] In addition, Fet3 catalyzes the reaction shown in eq 1.[9] The significance of this reaction to yeast biology is that Fet3 is absolutely required for high-affinity iron uptake in this organism.[7,10] The essentiality of the ferroxidase reaction to yeast iron metabolism lends support for the role of Cp and its ferroxidase activity in human iron homeostasis. Here we provide the spectral evidence which for the first time shows that Fet3 is a multinuclear Cu(II) protein and thus is a structural as well as functional homologue of Cp.

For our spectral analyses we produced in yeast a recombinant form of Fet3 that lacked the C-terminal transmembrane domain indicated by sequence analysis.[11] This protein, truncated at $\mathrm{Gly_{555}}$ but which retained all of the sequence elements associated with the putative Cu(II) binding sites,[7,8] was secreted as a strictly soluble protein. This protein was recovered directly from the growth medium in one step by adsorption to monoQ. Following elution and rechromatography on monoQ, approximately 4 mg/L of >95% pure Fet3 could be isolated. This protein was heavily glycosylated. Treatment with EndoH removed ∼70% of the carbohydrate; the resulting protein was 16% (w/w) hexose.[15] The UV−vis spectrum of the (partially) deglycosylated protein is shown in Figure 1; the spectrum of the untreated protein was identical. The spectrum shows the intense transition at ∼600 nm that gives the deep blue color to proteins, including this Fet3 preparation, that possess a type 1 Cu(II) site.[1,8] For Fet3, this transition occurs at 607 nm, $\epsilon = 5500\ \mathrm{M^{-1}\ cm^{-1}}$. The intensity of this transition is due to its strong charge-transfer character. In type 1 Cu(II) sites, this transition is due to Cys-S π to $\mathrm{Cu^{2+}}$ $d_{x^2-y^2}$ charge transfer.[8] The presence of this transition and its intensity are prima facie evidence for the presence of at least one type 1 Cu(II) in Fet3.[7]

Also evident in the spectrum shown in Figure 1 is a shoulder at ∼330 nm, $\epsilon = 5000\ \mathrm{M^{-1}\ cm^{-1}}$. This near-UV band is characteristic of the type 3 binuclear Cu(II) cluster found in laccase and ascorbate oxidase, for example.[2,3,8] The intensity of this transition is a function of the state of oxidation of the binuclear cluster. Addition of up to 30 equiv of $\mathrm{H_2O_2}$ had no effect on this band, indicating that, in the protein as-isolated, all of the apparent type 3 sites present in this preparation were fully oxidized.

Type 2 Cu(II) is "normal" in the sense that it exhibits only the weak d−d transitions characteristic of tetragonally distorted

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(11) Soluble Fet3 protein was produced from plasmid pDY148 in yeast strain M2 in which expression from the *FET3* promoter was high and independent of cellular [Fe].[12] To construct pDY148, the wild-type *FET3* locus in multicopy plasmid YEp352[13] was digested with *Age*I (5′ of the transmembrane domain) and *Pml*I (3′ of the transmembrane domain). The new, truncated 3′ end was ligated to an annealed double-stranded oligo, which encoded the FLAG epitope.[14]

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(15) Nanospray mass spectral analysis of the EndoH-treated protein yielded a major mass peak of 72972 kDa. Acid-anthrone[16] hexose analysis of this preparation showed that it contained 65 mol hexose/mol Fet3 or, on the basis of an average hexose mass of 180 g/mol, a total hexose mass/mol Fet3 of 11.7 kDa. This result was consistent with the difference between the mass peak (72972 kDa) and the protein M_r of 61.8 kDa predicted by the DNA sequence and N- and C-terminal analyses.

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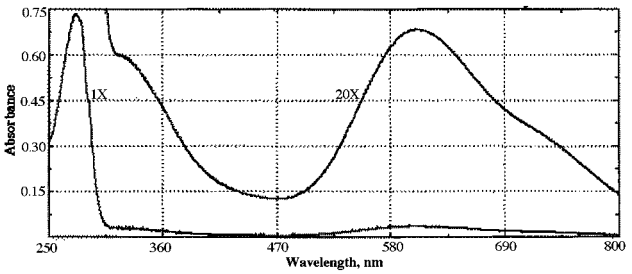

Figure 1. UV−vis spectrum of soluble, recombinant Fet3 protein. The spectra were recorded at room temperature on EndoH-treated Fet3 (followed by rechromatography on MonoQ) in MES buffer, pH = 6.0. For the visible and near-UV spectrum, the [Fet3] = 123 μM (20×); for the UV spectrum, this sample was diluted to [Fet3] = 6.2 μM (1×). The $A_{278nm}/A_{607nm} = 21.5 \pm 0.5$; for Cp, this value is 22.[4]

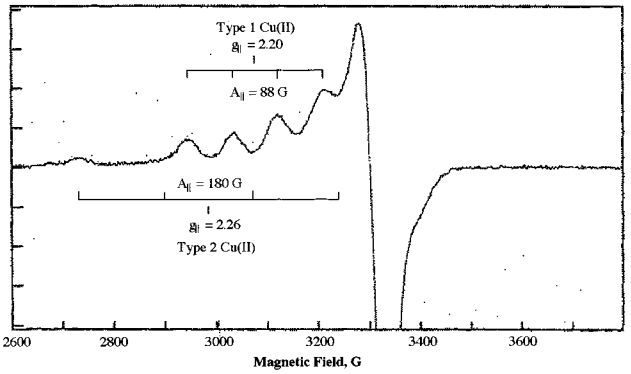

Figure 2. X-Band EPR spectrum of soluble, recombinant Fet3 protein. The spectrum was recorded on EndoH-treated protein in 50% v/v ethylene glycol/MES buffer, pH = 6.0 at 20 K. This sample contained 40 mg protein/mL or 0.65 mM Fet3. The instrument settings were the following: microwave frequency, 9.480 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 10 G; time constant, 0.02 s; sweep time, 60 s.

octahedral or nominally square planar Cu(II),[1,8] transitions that would be masked by the intense CT transition due to the type 1 site. However, type 1 and type 2 Cu(II) have readily distinguishable EPR spectra in that type 1 Cu(II) exhibits unusually weak Cu hyperfine coupling ($A_{||} = 40-90 \times 10^{-4}$ cm^{-1}) while type 2 Cu(II) exhibits a normal Cu hyperfine coupling of $160-200 \times 10^{-4}$ cm^{-1}.[8] This latter feature leads to the presence of an $M_I = +^3/_2$ type 2 Cu(II) low-field transition in the EPR spectrum clearly distinguishable from the hyperfine splittings due to a type 1 Cu(II) if present. Indeed, the CW X-band EPR spectrum of this recombinant Fet3 exhibited such a transition (at 2730 G, Figure 2). This spectrum, which is essentially identical to the EPR spectrum, of e.g., laccase,[2] demonstrated the features of both type 1 and type 2 Cu(II) with the following $g_{||}$ and $A_{||}$ values: 2.20, 91 $\times 10^{-4}$ cm^{-1} (type 1) and 2.26, 190 $\times 10^{-4}$ cm^{-1} (type 2), respectively. The $g_{\perp}$ value was 2.05.

Thus, the EPR spectrum confirms the presence of a type 1 Cu(II) as indicated by the visible absorbance, while it provides unique evidence for the presence in Fet3 of a type 2 Cu(II) as well. Integration of the spectrum indicated that the EPR-detectable Cu(II) sites were equally abundant in this sample. Note that a binuclear Cu(II) pair, if present, would be strongly antiferromagnetically coupled and therefore EPR silent.[8] Although a negative result, the lack of any feature in the EPR spectrum not attributable to either a type 1 or type 2 Cu(II) is consistent with the presence of a type 3 Cu(II) pair in Fet3 as was indicated by the 330 nm transition discussed above.

Amino acid compositional analysis of this recombinant Fet3 allowed us to determine the protein concentration of our preparations exactly.[15] Flameless atomic absorption spectrophotometry demonstrated that the only metal contained in Fet3 was Cu; 63 $\pm$ 2 nmol Cu/mg protein was determined. Furthermore, N- and C-terminal analyses, along with the deduced amino acid sequence, allowed the calculation of the precise M_r for this protein.[15] Thus, a precise Fet3 molar concentration could be calculated. This value, together with flameless atomic absorption analysis of the protein sample, enabled us to calculate a copper atom stoichiometry, or 3.9 gm atom Cu/mol Fet3. This value was consistent with the stoichiometry of EPR-visible versus EPR-silent Cu in that 2 Cu per enzyme molecule could be seen in the EPR spectrum while 2 Cu appeared to be EPR silent.

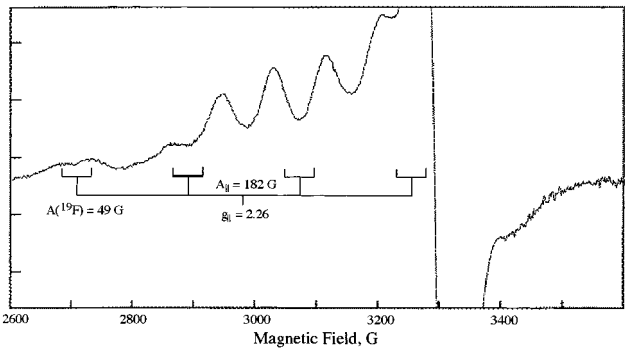

Figure 3. X-Band EPR spectrum of the F^- complex of soluble, recombinant Fet3 protein. The spectrum was recorded on EndoH-treated protein in 50% v/v ethylene glycol/MES buffer, pH = 6.0 containing 10 mM NaF. The [Fet3] = 0.56 mM. The instrument settings were the following: microwave frequency, 9.454 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 20 G; time constant, 0.16 s; sweep time, 167 s.

A functionally important feature of the type 2 Cu(II) in laccase, for example, is that exogenous ligands, including the substrate dioxygen, bind at this site. In the case of potentially bidentate ligands such as O_2 or N_3^-, the ligand bridges the type 2 Cu(II) with one of the type 3 Cu(II).[2,3,8] The apparent type 2 Cu(II) site in Fet3 conforms to this coordination chemistry in that upon addition of 10 mM F^-, the low-field EPR transition assigned to this Cu(II) is clearly split by a ^{19}F superhyperfine coupling (Figure 3). This value is 52×10^{-4} cm^{-1}, which can be compared to the values for this coupling in laccase[2] and ascorbate oxidase[3] of 54 and 57×10^{-4} cm^{-1}, respectively. In addition to the ^{19}F coupling observed in $g_{||}$, a significantly stronger coupling can be deduced in $g_{\perp}$. This is evident in the new feature centered at ~3400 G (Figure 3). Equatorial coordination of F^- to a type 2 Cu(II), e.g., in galactose oxidase,[17] can result in a strong superhyperfine coupling of up to 170×10^{-4} cm^{-1}. Binding of F^- to the type 2 Cu(II) in a type 1 Cu(II)-depeleted form of laccase[2] and in ascorbate oxidase[3] results in the appearance of a similar transition at ~3400 G in the X-band EPR spectra of each of these Fet3 homologues. Thus, not only does Fet3 appear to have a type 2 Cu(II), but the manner in which this site coordinates exogenous ligands appears to be equivalent to that associated with this type of Cu(II) in the other members of the multinuclear copper oxidase family.

In conclusion, we have provided spectroscopic evidence for the presence of type 1, type 2, and type 3 Cu(II) sites in Fet3. Further structural and functional characterization of this protein and of specific ligand and functional mutants in yeast will enable the elucidation of the origin of this protein's ferroxidase activity and of its role in iron uptake in this model eukaryotic organism.

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