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Cuprous oxidase activity of yeast Fet3p and human ceruloplasmin: implication for function

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Abstract The Fet3 protein in *Saccharomyces cerevisiae* and mammalian ceruloplasmin are multicopper oxidases (MCO) that are required for iron homeostasis via their catalysis of the ferroxidase reaction, $4Fe^{2+}+O_2+4H^+ \rightarrow 4Fe^{3+}+2H_2O$. The enzymes may play an essential role in copper homeostasis since they exhibit a strikingly similar kinetic activity towards Cu^{1+} as substrate. In contrast, laccase, an MCO that exhibits weak activity towards Fe^{2+}, exhibits a similarly weak activity towards Cu^{1+}. Kinetic analyses of the Fet3p reaction demonstrate that the ferroxidase and cuprous oxidase activities are due to the same electron transfer site on the enzyme. These two ferroxidases are fully competent kinetically to play a major role in maintaining the cuprous–cupric redox balance in aerobic organisms.

Key words: Ferroxidase; Ceruloplasmin; Fet3p; Copper homeostasis; Copper toxicity; Multicopper oxidase; *Saccharomyces cerevisiae*

1. Introduction

Ferroxidases are a group of multicopper oxidases (MCO) that exhibit an activity towards Fe^{2+} not shared by the other members of this family of copper proteins [1,2]. The copper proteins in this family are distinguished by having three distinct copper sites, Cu(II) types 1, 2 and 3. All of these enzymes couple the oxidation of four molecules of a one-electron donor substrate with the reduction of one molecule of dioxygen to $2H_2O$. The oxidation of the reducing substrate occurs at the type 1 Cu(II) while the reduction of O_2 occurs at the type 2, type 3 grouping of copper atoms known as the trinuclear cluster [2].

The substrate specificity of these enzymes is quite broad. For example, over 50 substrates have been examined in the reaction catalyzed by ceruloplasmin (Cp), a mammalian MCO that is abundant in the serum and in interstitial fluid, as in the brain [3–5]. One striking specificity that has been characterized is the activity that only some MCOs exhibit towards Fe^{2+} as reducing substrate. The oxidation by O_2 of ferrous iron is known as the ferroxidase reaction [1]. This catalysis is not restricted to this group of MCOs; it is exhibited also by ferritin in the process of iron core formation in this protein [6].

Cp and the yeast plasma membrane protein, Fet3p [7–10], are the best characterized ferroxidases, as these specific MCOs are called. Genetic studies in both mammals and yeast have established that the activity of these enzymes is essential to iron homeostasis in their respective organisms. For example, Cp knock-out mice exhibit the pathophysiology characteristic of the genetic disorder aceruloplasminemia; this disease is marked by severe neurologic deficits [3,11,12]. In yeast, the action of Fet3p on Fe^{2+} is coupled to the transport of the Fe^{3+} product into the yeast cell; Fet3p 'knock-out' yeast are therefore iron-deficient [7,13]. This 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 [3,4].

The pathophysiology in mammals associated with a deficit of Cp activity has commonly been attributed, in part, to an oxidative stress that results from a dys- or mislocalization of iron [3,14–16]. This inference is consistent with the reactivity of Fe^{2+}, in particular, in reaction with O_2 and its reduction products, most importantly, H_2O_2. The one-electron reduction by Fe^{2+} of O_2 produces the superoxide radical while the reaction with H_2O_2 produces the hydroxyl radical [17]. The latter species, in particular, is strongly cytotoxic. Cu^{1+} is equally adept at supporting the one-electron reduction (pro-oxidant) processes that produce these potentially damaging radical species [18]. Copper toxicity is well-known and, at least in part, it is associated with an oxidative stress, also [19]. Significantly, copper toxicity in yeast is exacerbated by the absence of Fet3p in the plasma membrane [20,21].

Recognizing that the lower valent forms of redox active metals like copper and iron are well-known as pro-oxidants due to their support of the one-electron redox chemistry noted above, we have considered the possibility that ferroxidases may play a protective as well as strictly iron metabolic role in aerobes. With iron, this cytoprotective role is difficult to separate from these enzymes' role in iron homeostasis per se. However, these enzymes have no comparable function in copper metabolism. Thus, demonstrating their activity towards Cu^{1+} as substrate would provide the basis for proposing that this activity is expressed in vivo and thereby plays a critical role in the suppression of copper and iron cytotoxicity. This cuprous oxidase activity is analytically characterized here for the first time.

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Abbreviations: MCO, multicopper oxidase; hCp, human ceruloplasmin; Hp, hephaestin; GPI, glycosyl phosphatidylinositol

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2. Materials and methods

2.1. Enzymes

Human ceruloplasmin (hCp) [22] and *Rhus vernicifera* laccase [23] were obtained from the Solomon laboratory (Stanford University, Palo Alto, CA, USA). Well-characterized, soluble forms of wild type Fet3p, and a type 1 Cu(II)-depleted Fet3p mutant, Fet3p (C484S), were purified as described [9,10]. All protein samples used in the following kinetic analyses were homogeneous as determined by sodium dodecyl sulfate gel electrophoresis.

2.2. Kinetic analyses

All kinetic analyses were based on oxygen consumption using an Oxygraph (Hansatech, www.hansatech-instruments.co.uk) [24]. Rates of O_2 uptake were evaluated using the OXYG32 software provided by Hansatech. All initial velocity, v versus [S] data were subsequently analyzed by direct fitting to the Michaelis–Menten equation using Prism software from GraphPad (GraphPad Software, San Diego, CA, USA). Ferrous ammonium sulfate (Sigma, St. Louis, MO, USA) was used for kinetic analysis of the ferroxidase reaction; cuprous chloride (Alfa Aesar, Ward Hill, MA, USA) was used to measure cuprous oxidase activity. The stock solution of the former substrate was freshly prepared in nitrogen-purged buffer; the stock solution of the latter substrate was in acetonitrile. All transfers from these stock solutions were done using gas-tight syringes. The buffer used for O_2 uptake measurements was air-saturated, pH 5.0, 0.1 M MES that, in all cases, contained 5% acetonitrile (final v/v percent). Laccase was assayed also with *p*-phenylenediamine (Sigma) as a check on its oxidase activity. This substrate was recrystallized before use and stored under N_2. To test whether Cu^{1+} and Fe^{2+} were being turned over at the same active site in Fet3p, O_2 uptake measurements were made in mixtures of the two substrates at various ratios. These measured velocities were then compared to the velocities predicted for single active site versus two active sites kinetic models [25]. These predicted velocities were generated in the program Origin (OriginLab, Northampton, MA, USA) using the kinetic constants determined in this work (Table 1).

3. Results

3.1. Steady-state kinetic analysis of the cuprous oxidase reaction catalyzed by Fet3p and hCp

A standard v versus [S] analysis of the oxidation of cuprous ion by these two ferroxidases was carried out. The experimental data for the Fet3p reaction are given in Fig. 1A (closed circles); the curve represents the theoretical fit based on the fitted kinetic constants given in Table 1. The data for the hCp-catalyzed reaction behaved comparably as shown in Fig. 1A (open circles); the fitted values for the kinetic constants for this reaction are given in Table 1, also. The reaction mixtures with Cu^{1+} contained 5% (v/v) acetonitrile, the solvent for stock solutions of cuprous chloride. Consequently, the steady-state ferroxidase kinetics for Fet3p and hCp were determined in the same solvent mixture. These fits are shown in Fig. 1B (closed and open circles, respectively). The corresponding sets of fitted kinetic constants are tabulated in Table 1, also.

In contrast to the two ferroxidases, even at 300 μM Cu^{1+}, laccase exhibited little activity with this substrate (Fig. 1A, open triangles). This [Cu^{1+}] was equivalent to at least 9K_M for either of the ferroxidases (Table 1) indicating that for laccase, the K_M for Cu^{1+} was ca. 1 mM or greater, or the k_{cat} was ca. 2 min^{-1} or less, based on the sensitivity of our O_2-electrode system. Laccase was essentially without activity towards Fe^{2+}, also (Fig. 1B, open triangles). Assay of the laccase using *p*-phenylenediamine as substrate demonstrated that the enzyme preparation used had an oxidase activity comparable to that reported in the literature (data not shown). These data indicate that the ferroxidases, specifically, share a cuprous oxidase activity that is not exhibited by an MCO known to have a strongly diminished reactivity with Fe^{2+} as substrate [10].

Table 1
Kinetic constants for the cuprous oxidase and ferroxidase reactions of Fet3p and hCp

Enzyme	K_M (μM)		k_{cat} (min^{-1})	
	Cu^{1+}	Fe^{2+}	Cu^{1+}	Fe^{2+}
Fet3p	37.9 ± 3.6	5.4 ± 0.8	79.2 ± 2.7	63.9 ± 2.5
hCp	36.8 ± 5.1	8.3 ± 1.5	22.5 ± 0.8	30.3 ± 1.6

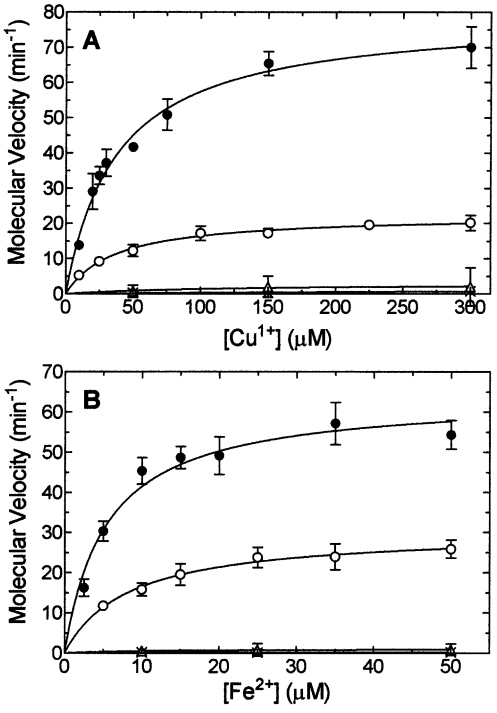

Fig. 1. Steady-state kinetic analysis of cuprous oxidase and ferroxidase reactions catalyzed by Fet3p, hCp and laccase. A: The cuprous oxidase reaction: wild type Fet3p, closed circles; hCp, open circles; laccase, open triangles; Fet3p(T1D) mutant, crosses. B: The ferroxidase reaction: Fet3p, closed circles; hCp, open circles; laccase, open triangles; Fet3p(T1D) mutant, crosses. Note that the experimental values are given in *molecular* velocities, $v_{measured}/[E]$. This normalizes the measured velocities so that they can be directly compared among the various enzyme species used. These data were fit directly to the Michaelis–Menten equation generating the traces shown in the figure and the fitted values for K_M and k_{cat} that are given in Table 1.

3.2. The ferroxidase and cuprous oxidase reactions catalyzed by Fet3p occur at the same enzyme active site

To demonstrate that the apparent metallo-oxidase activity of Fet3p originated from one redox site, specifically, the type 1 Cu(II), two experiments were performed. First, kinetic competition between Cu^{1+} and Fe^{2+} was determined in the Fet3p reaction by measuring O_2 uptake as a function of [Cu^{+1}] and [Fe^{2+}] in the reaction mixture. Briefly, if two substrates are turned over at the same site, the velocity measured in a mixture of the two will be less than the sum of the velocities measured if each is present separately. This is because of the substrate competition due to one of the substrates on the measured velocity due to the other. In contrast, if the sub-

strates are turned over at separate sites, then the velocity from a mixture will equal this sum irrespective of the substrates' concentrations. These two possibilities are analytically described by Eqs. 1 and 2, respectively [25].

$$V_t = \frac{V_{\max Fe}\,[Fe^{2+}]}{K_{m\,Fe}(1+[Cu^{1+}]/K_{m\,Cu})+[Fe^{2+}]} + \frac{V_{\max Cu}\,[Cu^{1+}]}{K_{m\,Cu}(1+[Fe^{2+}]/K_{m\,Fe})+[Cu^{1+}]} \quad (1)$$

$$V_t = \frac{V_{\max Fe}\,[Fe^{2+}]}{K_{m\,Fe}+[Fe^{2+}]} + \frac{V_{\max Cu}\,[Cu^{1+}]}{K_{m\,Cu}+[Cu^{1+}]} \quad (2)$$

The results of this analysis are given in Fig. 2. The experimental points (open circles) are compared to the first of these models, Eq. 1 (single site, lower curve) and to the second of these models, Eq. 2 (two sites, upper curve). Clearly, the data conform to the former, same site model indicating that the two metal ions are turned over competitively at the same redox site on the enzyme. To emphasize: what distinguishes this model from simple, two site ones is that turnover in the presence of both substrates is always *less* than that in the presence of only one when at the same *total* concentration. This concentration dependence arises distinctly from the $(1+[Me^{2+}]/K_{Me})$ terms in the denominators in Eq. 1 that describe the mutually exclusive binding of the two metal ions to a single active site on the enzyme.

This site is well-known to be the type 1 Cu(II). For example, a mutant of Fet3p, C484S, lacks the type 1 Cu(II) since C484 is one of the ligands to the copper at this site [10]. This type 1-depleted Fet3p (designated Fet3pT1D, see [10]) is inactive with Fe^{2+} as substrate (Fig. 1B, crosses, and [10]); it is inactive with Cu^{1+} as well (Fig. 1A, crosses). Other copper site-depleted mutant forms of Fet3p [10,26,27] were similarly

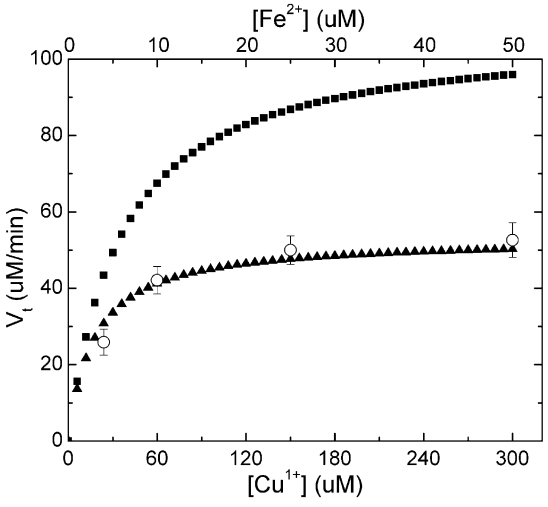

Fig. 2. Steady-state kinetic analysis of Cu^{1+}, Fe^{2+} competition in the Fet3p reaction. O_2 uptake data were obtained as described in Fig. 1 using the mixtures of $[Cu^{1+}]$ and $[Fe^{2+}]$ indicated in the plot. The theoretical curves for Eqs. 1 and 2 (text) were generated in Origin (OriginLab) using the kinetic constants for the ferroxidase and cuprous oxidase reactions given in Table 1 for Fet3p. The lower curve is for Eq. 1, the case in which the two substrates react competitively at the same site; the upper curve is for Eq. 2, the case in which the two substrates react independently at two separate, non-interacting sites [25].

inactive with both Fe^{2+} and Cu^{1+} as substrate (latter data not shown). Thus, the cuprous oxidase activity of Fet3p is not obviously due to some non-specific site on the protein with kinetics that fortuitously mimic how single site kinetics would behave in the competition experiments above.

4. Discussion

The ferroxidases Fet3p and hCp are robust catalysts of the oxidation of cuprous to cupric copper. The only strong difference in this cuprous oxidase reaction in comparison to the ferroxidase one is that the K_M for Cu^{1+} is generally larger for these two ferroxidases. The inferred weaker binding of Cu^{1+} in comparison to Fe^{2+} can be ascribed to the +1 charge difference in the latter substrate. The binding of metal cations to hCp has been localized to a region of the protein that presents an electrostatic surface that is overall negative, binding that has been ascribed specifically to a coordination sphere that includes a preponderance of acidic amino acid side chains [28]. Consistent with the difference in K_M values for Fe^{2+} and Cu^{1+} in the hCp reaction is the fact that in the hCp crystal, copper bound to this region of the protein is 'labile' and is displaced by iron. Mutagenesis studies in Fet3p have implicated two acidic residues in Fe^{2+} binding to this protein as well [24,29]. In contrast, laccase has a substrate binding site hospitable for large, bulky aromatic reductants, not for a di- or trivalent metal ion [30]. This structural difference can account, at least in part, for the specificity that hCp and Fet3p in comparison to laccase exhibit, a specificity that we now have shown extends to Cu^{1+}.

As noted, there are no data that suggest a role for catalysis of Cu^{1+} oxidation in copper metabolism comparable, for example, to the essential role that Fet3p and its orthologs play in high affinity iron uptake in yeasts [31] and, apparently, in photosynthetic green algae [32]. In fact, high affinity copper uptake in eukaryotes appears to rely most heavily on the Ctr1 protein that, in turn, appears to use Cu^{1+} as substrate [33]. However, the Cu^{1+} for uptake is most likely generated in situ by the reduction of environmental Cu^{2+} catalyzed by a plasma membrane metalloreductase. In *S. cerevisiae* this is the Fre1 protein [34]; in the mammalian epithelial cell, this would be the Dcytb protein [35].

That is, eukaryotes are producing quantities of Cu^{1+} in the course of normal copper metabolism. As an enzyme product, this Cu^{1+} has the potential to equilibrate with bulk solvent, that is, it can be considered 'free'. This certainly is the case in the yeast Cu^{2+} reduction/copper uptake system where cuprous copper for uptake can come directly from the bulk solvent, and not necessarily from Fre1p [36]. In short, eukaryotic cells are normally producing a fairly aggressive pro-oxidant. Our data demonstrate that the cuprous oxidase activity of Cp in mammals, or of the Fet3 protein in yeast, is *kinetically* competent to maintain cuprous ion 'homeostasis' with regard to this pro-oxidant's steady-state concentration. Note, however, that this picture applies to a cell's 'extracellular' compartment, not to any intracellular one. O'Halloran and his co-workers have examined in some detail the state of intracellular copper and have concluded that there is little if any 'free' copper [37]. In contrast to the situation at the cell surface, copper in cells is essentially all protein-bound, e.g. to copper chaperones and, in copper excess, to metallothioneins. In short, our data suggest that the eukaryotic cell has two lines of defense with

regard to copper: one that is redox-based to deal with 'free', environmental copper, and one that is chelator-based, for metabolic copper in the cell.

The CueO protein, an MCO from *Escherichia coli* that is encoded within a copper resistance operon in the genome of this bacterium, is a ferroxidase localized to the periplasmic space that exhibits a pattern of substrate reactivity similar to that recorded for Cp [38–40]. Three groups working with this protein and its encoding *cue* system (for Cu efflux) have suggested that CueO (previously known as the *yakK* gene product) could contribute to copper resistance by acting as a cuprous oxidase thus shifting the Cu^{2+}/Cu^{1+} ratio towards Cu^{2+}. In this model, the cupric ion would be less readily taken into the cell in addition to its being a weaker pro-oxidant, thus explaining the cytoprotective effect of the CueO protein. However, one of these groups reported that despite a substrate reactivity profile comparable to that of hCp, including its reactivity with Fe^{2+}, CueO exhibited no oxidase activity with Cu^{1+} as substrate [40]. Therefore, the role of cuprous oxidase activity in copper homeostasis in prokaryotes remains unresolved.

On the other hand, two labs have reported that deletion of *FET3* in *S. cerevisiae* results in a copper sensitivity not seen in wild type yeast [20,21]. One group suggested that as a copper protein, Fet3p might play some role in sequestering copper, thus suppressing its cytotoxic potential [21]. The other researchers noted that the expression of low affinity metal transporters was increased in a Δ*fet3* strain. Therefore, they suggested that the increased copper sensitivity in this strain might be due to an increased copper accumulation via these other pathways [20]. Neither group considered the possibility that Fet3p played a *catalytic* role in maintenance of copper homeostasis. The kinetic analyses described here provide evidence in support of this possibility.

In fact, recent data from the Zhu lab appear to link the cuprous oxidase activity that we have described here with protection against copper stress in yeast [41]. Zhu and his co-workers demonstrated that the copper sensitivity exhibited by yeast strains carrying a deletion of the *FET3* gene was suppressed in this background by deletion of the *FRE1* gene that encodes the predominant cell surface metalloreductase in *S. cerevisiae*. In contrast, over-production of Fre1p and thus of plasma membrane Cu^{2+} reductase activity strongly increased the copper sensitivity of Δ*fet3* as well as wild type yeast strains. Since this copper sensitivity was completely independent of copper uptake and, therefore, of abnormal *intracellular* copper, these workers concluded that *extracellular*, Fre1p-generated Cu^{1+} was the dominant cytotoxic agent in copper sensitivity. In turn, the steady-state level of this Cu^{1+} was reduced by its oxidation to Cu^{2+} by Fet3p via the cuprous oxidase reaction characterized in this report.

The suggestion that Fet3p plays a specific physiologic role in yeast copper metabolism via its four-electron reduction of O_2 to water using $4Cu^{1+}$ as substrate is predicated on the assumption that there is a significant steady-state level of reduced metal species at the cell surface due to the Fre1p reaction. This situation would require that the relative velocities of the two processes involved in copper uptake – reduction and transport – were $V_R > V_T$. In yeast, these reduction and uptake values are 7 and 0.7 nmol Cu/min/mg cell protein, respectively [36] indicating that there is 'excess' Cu^{1+} produced as pro-oxidant and as potential substrate for Fet3p. This 'excess' is increased ca. eight-fold in yeast strains that over-produce Fre1p; as noted, these strains exhibit a correspondingly exaggerated sensitivity to copper [36].

The fact that hCp also exhibits cuprous oxidase activity argues that this protein and its homolog, hephaestin (Hp), could play a comparable copper-protective role in mammals. There are three ferroxidases in mammals: the soluble, secreted Cp; a glycosyl phosphatidylinositol-anchored form of Cp (GPI-Cp) identified in the brain [42], retina [43] and Sertoli cells [44]; and Hp, a type 1 membrane protein found in the intestinal epithelium [45]. Based on their homology and on the results here, the latter two proteins, like Cp itself, are most likely cuprous oxidases, also. Thus, all three may well contribute to the suppression of nascent copper toxicity in mammals due to the accumulation of Cu^{1+}. Of note is the fact that increased oxidative stress is a cellular phenotype linked to aceruloplasminemia. This stress has been described in brain [46] and retina [47] while oxidative stress in the latter tissue associated with photic injury is linked to a strong induction of mRNA for both the soluble and GPI-linked forms of Cp [43]. Also, GPI-Cp is anchored to the *extracellular* face of the plasma membrane, an orientation identical to that of Fet3p, and, therefore, is well-positioned to scavenge Cu^{1+} produced at or near this membrane surface [44].

Cu^{1+} is a pro-oxidant comparable in activity to Fe^{2+}. One example of this is the fact that in complex with ADP, Cu^{2+} exhibited 70% of the activity of Fe^{3+} in the peroxidation of rat liver microsomal phospholipids with dopamine and dioxygen supporting the catalytic redox cycle [14]. This type of lipid peroxidation has been proposed as a possible mechanism of *iron* neurotoxicity, as is implied in the neural pathophysiology observed in aceruloplasminemia [11,12,15,16]. In fact, Cp blocked lipid peroxidation in this in vitro assay with Fe^{3+}-ADP as catalyst [14]. Unfortunately, its effect on the Cu^{2+}-ADP-catalyzed reaction was not reported. On the other hand, in a brief report on the pathophysiology in patients with aceruloplasminemia, Gitlin and co-workers may have demonstrated a protective role for hCp in *cuprous* ion-catalyzed lipid peroxidation [48]. However, to date, there are no *explicit* reports of studies on possible mechanisms of the *copper* neurotoxicity that would reasonably follow in Cp deficiency if Cp and its congeners played an essential role in copper homeostasis as cuprous oxidases. Hopefully, the results described here, and those reported by Shi et al. [41], will encourage others to embark on such studies.

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