Copper-only superoxide dismutase enzymes and iron starvation stress in Candida fungal pathogens

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Copper (Cu)-only superoxide dismutases (SOD) represent a newly characterized class of extracellular SODs important for virulence of several fungal pathogens. Previous studies of the Cu-only enzyme SOD5 from the opportunistic fungal pathogen Candida albicans have revealed that the active-site structure and Cu binding of SOD5 strongly deviate from those of Cu/Zn-SODs in its animal hosts, making Cu-only SODs a possible target for future antifungal drug design. C. albicans also expresses a Cu-only SOD4 that is highly similar in sequence to SOD5, but is poorly characterized. Here, we compared the biochemical, biophysical, and cell biological properties of C. albicans SOD4 and SOD5. Analyzing the recombinant proteins, we found that, similar to SOD5, Cu-only SOD4 can react with superoxide at rates approaching diffusion limits. Both SODs were monomeric and they exhibited similar binding affinities for their Cu cofactor. In C. albicans cultures, SOD4 and SOD5 were predominantly cell wall proteins. Despite these similarities, the SOD4 and SOD5 genes strongly differed in transcriptional regulation. SOD5 was predominantly induced during hyphal morphogenesis, together with a fungal burst in reactive oxygen species. Conversely, SOD4 expression was specifically up-regulated by iron (Fe) starvation and controlled by the Fe-responsive transcription factor SEF1. Interestingly, Candida tropicalis and the emerging fungal pathogen Candida auris contain a single SOD5-like SOD rather than a pair, and in both fungi, this SOD was induced by Fe starvation. This unexpected link between Fe homeostasis and extracellular Cu-SODs may help many fungi adapt to Fe-limited conditions of their hosts.

Superoxide dismutase (SOD)2 enzymes catalyze the dismutation of superoxide anion-free radical into hydrogen peroxide and molecular oxygen, and thereby play a key role in detoxification and metabolism of reactive oxygen species (ROS). These enzymes can either be intracellular or extracellular, and have evolved to utilize different metal cofactors (1). SOD enzymes can participate in signaling pathways involving ROS and also protect cells from superoxide toxicity (1). Intracellular SODs in particular prevent superoxide damage to Fe-S cluster containing enzymes (2). Extracellular SODs can also guard against superoxide toxicity, a prime example being the extracellular SODs of microbial pathogens thwarting the oxidative attack of host immunity (3). However, the targets of superoxide damage with extracellular SODs are poorly understood, as Fe-S cluster enzymes are generally not considered to be extracellular.

We recently identified a new class of extracellular SODs, namely eukaryotic Cu-only SODs that are unique to fungi and fungal-like oomycetes (4–6). Cu-only SODs are closely related to the ubiquitous family of Cu/Zn-SODs, but are missing a Zn co-factor and electrostatic loop sequences of Cu/Zn-SOD (4) believed to act in substrate guidance (7–9). Unlike Cu/Zn-SODs, which are both intra- and extracellular, Cu-only SODs are exclusively extracellular and seem to be largely attached to the cell surface through GPI anchors (10, 11). Bioinformatic analysis indicates that Cu-only SODs are the only extracellular SODs of the fungal kingdom, and are present in fungal pathogens and nonpathogens across different phyla (5).

With several fungal pathogens including Candida albicans (10, 12, 13), Paracoccidioides spp. (14), Histoplasma capsulatum (15), and Beauveria bassiana (16), Cu-only SODs have been shown to protect the microbe against the oxidative burst of the host immune response. Currently, much of what we know regarding the biology of Cu-only SODs has emerged

2 The abbreviations used are: SOD, superoxide dismutases; ROS, reactive oxygen species; YPD, yeast extract, peptone, dextrose-based medium; BPS, bathophenanthroline disulfonic acid; AAS, atomic absorption spectrophotometry; PAR, 4-(2-pyridylazo)resorcinol; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolum; ICP-MS, inductively coupled plasma mass spectrometry; RLU, relative luminescence units; BCS, bathocuproine disulfonate; DPI, diphenylenedioneum; PNGase F, peptide N-glycosidase F; TEV, tobacco etch virus; qRT, quantitative RT; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxyethyl)propane-1,3-diol.
Fungal Cu-only SODs, ROS, and iron starvation

Results and discussion

The biochemical and biophysical properties of recombinant SOD4 closely parallel SOD5

To compare the biochemical and biophysical properties of C. albicans SOD4 and SOD5, recombinant proteins missing the N-terminal signal peptide and C-terminal GPI anchor motif were produced in an Escherichia coli expression system. Homogeneous preparations of SOD4 and SOD5 were obtained using purification schemes that were identical except that SOD4 was seen to bind anion exchange resin under conditions where SOD5 did not (Fig. 1A), suggestive of different surface charge. Indeed, using modeling programs, SOD4 is predicted to have a more negatively charged surface on one face of the protein (Fig. 1B).

Cu-only SOD5 is a monomeric enzyme compared with dimeric or tetrameric Cu/Zn-SOD of eukaryotes (4, 28). Based on the differential surface charges of SOD4 versus SOD5 (Fig. 1B) we tested whether SOD4 is also monomeric or could form higher order complexes. In analytical ultracentrifugation studies (Fig. 1C) SOD4 and SOD5 show similar sedimentation coefficient values of 1.9 S and 1.8 S, consistent with both proteins being monomeric. Given their complementary surface charge profiles, we tested whether SOD4 and SOD5 could form heterodimers or higher order mixed complexes. However, as seen in Fig. 1C, bottom panel, the proteins retained their monomeric properties when combined in solution, and do not form stable complexes.

To compare the catalytic efficiencies of SOD4 and SOD5, the proteins reconstituted with Cu were subjected to pulse radiolysis methods for kinetic measurements of SOD activity. SOD5 was previously shown to react rapidly with superoxide in a concentration-dependent manner (4) and as seen in Fig. 2A, the same is true for Cu-only SOD4. At pH 7.25, the calculated second-order rate constant for SOD4 is $4.2 \times 10^4 \text{M}^{-1}\text{s}^{-1}$, a rate that closely approximates that of SOD5 (Fig. 2, B and C) (4, 5). Cu-only SOD5 is known to lose activity at pH $\geq 8.0$ compared with bimetallic Cu/Zn-SODs that have a much wider pH optimum due in part to the Zn co-factor (4, 29). Like SOD5, SOD4 reactivity with superoxide is greatly diminished at alkaline pH (Fig. 2C). We additionally tested the impact of ionic strength on SOD4 catalysis. With Cu/Zn-SOD1 and Cu-only SOD5, electrostatic interactions are believed to guide the superoxide substrate to the active site and as such, catalytic rates decrease with increasing ionic strength (4, 30–32). SOD4 shares this feature, we observed that SOD4 catalysis is also inhibited by increasing ionic strength (Fig. 2D), indicative of substrate guidance to the active site. Altogether, these pulse radiolysis studies demonstrate no significant difference in the SOD activity of the two C. albicans Cu-only SODs. Both are capable of disproportionating superoxide over acidic to neutral pH conditions with rates that approach diffusion limits.

It is noteworthy that in addition to the single catalytic Cu ion in SOD4, additional Cu can associate with the recombinant protein when Cu reconstitution is carried out under acidic or near neutral pH conditions. With independent preparations of the protein, SOD4 protein bound between 1.6 and 3.0 eq of Cu/mol of protein when reconstituted at pH 5.5 or 6.9. How-
ever, this additional Cu does not appear catalytically active. As seen in Fig. 2C, there is no increase in SOD activities with a SOD4 preparation containing 2.0 mol of Cu/mol of SOD compared with a SOD4 preparation containing 1.6 eq of Cu. We have previously observed additional Cu equivalents (noncatalytic) associated with a mutant allele of SOD5 (5), and it is possible this additional Cu association is a consequence of in vitro Cu reconstitution of the recombinant proteins. In any case, this additional Cu can be removed upon extensive dialysis at alkaline (8.0) pH. SOD4 bound to ≈1.0 eq of Cu in this manner was used for analytical ultracentrifugation experiments (above) and Cu binding stability constants described below.

We previously reported that *C. albicans* SOD5 binds Cu with a lower affinity compared with Cu/Zn-SOD1 and is sensitive to a wide array of metal chelators (23). We tested whether the same was true for SOD4. To focus solely on Cu bound to the active site of SOD4, we used a preparation of SOD4 with roughly 1:1 Cu-binding stoichiometry and activity that parallels Figure 1.

*C. albicans* SOD4 and SOD5 are monomeric proteins with differential surface charge. A, recombinant SOD4 and SOD5 was purified from *E. coli* inclusion bodies as described under "Experimental procedures," and during the indicated steps of purification, samples were analyzed by denaturing nondenaturing gel electrophoresis and Coomassie staining. Pre TEV, refolded protein isolated on Ni column; Post TEV, protein following removal of His tag by TEV protease; Ni FT, flow-through from HisTrap column to remove His tag and TEV; QFF FT and QFF elution, flow-through and bound fractions, respectively, from QFF anion exchange. Molecular mass markers (kDa) are shown on left. B, shown are SOD4 structural predictions using MODELLER and SOD5 PDB 4N3T as model, and electrostatic surface charges where scale ranges from −5 (red) to +5 (blue) in units of kBT/ec at pH 8.0. Top and bottom represent two faces of the protein, yellow circle denotes location of Cu site. C, analytical ultracentrifugation experiments of recombinant SOD4 (top), SOD5 (middle), or a mixture of the two (bottom) are consistent with monomeric versions of the proteins. Results are representative of two experimental trials with independent preparations of SOD proteins.
SOD5 with a similar Cu-binding stoichiometry (Fig. 3A). As seen in Fig. 3B, this SOD4 was susceptible to loss of its Cu co-factor in the presence of excess EDTA and exhibits a t_{1/2} of Cu binding under these conditions (6 h) that closely parallels SOD5 (23). By comparison, Cu/Zn-SODs stably bind Cu >24 h under the same conditions (23, 29, 33). Because we added EDTA in the pulse radiolysis experiments of Fig. 2, we controlled for any Cu loss by comparing the rates for the first pulse and the last pulse to ensure that activity was not lost during the time of the experiment. The stability constants for SOD4 binding to Cu were determined through equilibrium dialysis in the presence of 4-(2-pyridylazo)resorcinol (PAR) and glycine, and were found to be log K/Cu = 14–15, closely approximating published values for SOD5 (23) (Fig. 3C). Collectively, our studies with recombinant proteins show C. albicans SOD4 and SOD5 as highly similar SOD enzymes. Both are monomeric SODs with similar catalytic efficiencies and Cu-binding properties.

The differential expression of SOD4 versus SOD5 in C. albicans

Although the recombinant enzymes are highly comparable, it was important to examine SOD4 and SOD5 in their native fungal organism. As mentioned above, SOD5 is specifically transcriptionally induced in hyphal forms of C. albicans where it reacts with extracellular superoxide produced by the FRE8 NOX of this yeast (13, 17, 34, 35). Accordingly, we observed an ~30-fold increase in SOD5 and FRE8 transcript levels under hyphal inducing conditions. Notably, under these same conditions, SOD4 transcript levels were induced by no more than 2-fold (Fig. 4A).

Under what conditions is SOD4 transcriptionally induced? In previous proteomic studies, SOD4, but not SOD5 protein levels were seen to increase in a mixed population of C. albicans yeast-form and hyphal cells under Fe-starvation conditions (36). However, it is unclear whether SOD4 mRNA is induced by Fe starvation, as reports have been conflicting (37, 38). To investigate the possible regulation of SOD4 expression by Fe limitation, we comparatively examined SOD4 and SOD5 expression at both the RNA and protein levels in C. albicans starved for Fe by growth in the presence of the Fe chelator, bathophenanthrolinedisulfonic acid (BPS). Using a concentration of BPS that dramatically reduces intracellular Fe levels (Fig. 4B), we found that SOD4 mRNA is dramatically up-regulated over 100-fold by Fe restriction (Fig. 4C). SOD4 is induced by Fe starvation in yeast-form cells along with other genes known to be up-regulated by Fe restriction, including RBTS encoding the heme receptor (Fig. 4C). SOD4 is also abundantly expressed in hyphal cells with limited Fe (see Fig. 5, A and B). Compared with the dramatic >100-fold induction of SOD4 by Fe starvation, SOD5 mRNA was induced ~10-fold under the same conditions.
Figure 3. Cu-binding properties of *C. albicans* SOD4 compared with SOD5. A, recombinant SOD4 and SOD5 were subject to SOD activity measurements by WST-1 reduction (23). Results show triplicate measurements, representative of two experimental trials. B and C, top, equilibrium dialysis was carried out in the presence of an excess of the indicated metal chelators. At the indicated time points, the percent of Cu remaining in the SOD-containing samples was analyzed by AAS. Results are duplicate measurements from each of two independent dialysis experiments. C, bottom, the log K stability constants for Cu binding were determined using nonlinear fits to the curves in C, top, as described (23).

Figure 4. SOD4 and SOD5 are differentially expressed in *C. albicans* cultures. A, *C. albicans* was grown as either yeast-form cells (Yeast) or induced to form hyphae in IMDM for 2 h prior to qRT-PCR analysis of the indicated genes. Results show fold-changes in mRNA levels in hyphal over yeast-form cells; bars represent mean ± S.D. for three independent cultures. ***, p ≤ 0.0004; **, p = 0.0013. B–D, yeast-form cells were grown in the presence or absence of 150 μM of the Fe chelator BPS and were: B, analyzed for Fe content by ICP-MS, or C and D, subjected to qRT-PCR analysis of the indicated genes (C) or SOD4 (D), where results show fold-change in mRNA in + BPS over −BPS conditions. Results are shown for three (D) or 5–9 (B and C) independent cultures over two or more experimental trials; errors are S.D. **, p = 0.0167; ***, p = 0.0001; ****, p < 0.0001. Statistical significance was determined by unpaired t test. E, cells were induced to make hyphae overnight in IMDM under Fe-replete conditions (top), or yeast-form cells were made Fe-starved by growth in 150 μM BPS (bottom). Proteins covalently attached to the cell wall were liberated by lyticase, deglycosylated with PNGase F, and assayed by immunoblot using an anti-SOD4 antibody that cross-reacts with both SOD4 and SOD5. Additional details are provided in the legend to Fig. 5. Molecular mass markers in kDa are indicated on the left. Strains used: A–C, and E, WT, SCS314; D, WT SN152 and isogenic sef1Δ/Δ null; SS101, sod4Δ; SS100, sod5Δ; SS102, sod4Δ.
site was previously identified in sequences upstream of *C. albicans* SOD4 through ChIP-seq (37), and as seen in Fig. 4D, SOD4 transcriptional induction by Fe starvation is drastically reduced by the sef1Δ/Δ mutation. Thus, SOD4 appears to be part of the SEF1 regulon for the Fe starvation response of *C. albicans*.

It is important to note that although BPS has the capacity to chelate Cu(II), BPS does not create a Cu starvation state in the fungus. Total Cu levels associated with *C. albicans* are not lowered with BPS and if anything, are slightly elevated, albeit no more than 2-fold (Fig. S3A). Moreover, intracellular Cu/Zn-SOD1 that is an excellent biomarker of Cu availability (42, 43) does not change in either activity nor protein levels with BPS, unlike cells treated with the Cu(II) chelator bathocuproine sulphonate (BCS) (Fig. S3, B and C). The induction of SOD4 indeed reflects Fe and not Cu starvation, underscored by its control through SEF1 (Fig. 4D).

Although SOD5 and SOD4 are clearly differentially expressed at the level of mRNA, it was important to confirm this at the protein level. Using β1,3-glucanase (lyticase) to release cell wall GPI anchor proteins, we identified SOD4 and SOD5 proteins in cell wall fractions by immunoblot using an anti-SOD4 antibody that cross-reacts with both proteins (Fig. S1). Using WT and sod4Δ and sod5Δ mutant strains (Fig. 4E), we show that SOD5 but not SOD4 protein, is abundantly present in cell walls from hyphal cells (Fig. 4E, top), whereas only SOD4 protein is detected in Fe-starved yeast-form cells (Fig. 4E, bottom).

Altogether, these expression studies demonstrate that despite virtually identical biochemical properties, SOD4 and SOD5 have evolved to operate under distinct cellular conditions. SOD5 is highly induced in hyphal cells when cells produce a large burst of ROS, whereas SOD4 is strongly induced under Fe-starvation conditions irrespective of cell morphology.

### Localization of SOD4 and SOD5

Given that SOD4 and SOD5 are differentially expressed in *C. albicans*, we addressed whether their localization might also diverge. GPI-anchored proteins in *C. albicans* can be in the cell wall, secreted or attached to membranes (11, 44). All three locations may be relevant to the Cu-only SODs, as their superoxide substrate can come from exogenous sources, i.e. host immune cell NOX (12, 15, 45), or can be derived from the fungus itself through FRE8 NOX (17).

To compare SOD4 and SOD5 localization, cells were grown under conditions where both proteins were expressed (Fe-starved hyphal cells), and cell fractions analyzed using the aforementioned SOD4 antibody as well as an anti-SOD5 antibody that shows strong specificity for SOD5 (Fig. S1). In addition to abundant cell wall localization (Fig. 5A, lanes 5–8), both SODs were found in the growth medium (lanes 1–4), consistent with previous proteomic studies of the fungal cell wall and secretome (46–52). It is worth noting that although the cell wall fraction exhibited the anticipated ≈20 kDa molecular mass of SOD4 and SOD5, the secreted SODs were larger, ≈30 kDa. These larger species appear to represent SOD retaining a glucan modification, because SOD5 treatment with β1,3-glucanase (lyticase) yielded the expected 20-kDa species (Fig. S4). GPI-anchored proteins can be released during remodeling of
the cell wall (44, 53) and the glucan-modified SOD in the extracellular media may be the product of such remodeling. Secretion of glucan-modified proteins has been observed for other *C. albicans* GPI anchor proteins (54). In any case, release of Cu-only SODs into the extracellular environment may be beneficial for *C. albicans* in counteracting the superoxide burst of host immune cells.

*C. albicans* SOD4 and SOD5 contain the C-terminal serine/threonine-rich domain for GPI attachment to the cell wall (55) but lack the dibasic residues immediately before the ω site that signal plasma membrane localization in some GPI anchor proteins (56, 57). However, certain GPI anchor proteins that lack these dibasic residues still localize in membranes, e.g. *C. albicans* RBT5 (58, 59). We therefore tested whether a portion of SOD4 and SOD5 are membrane-localized using an SDS extraction procedure described for RBT5 (59). As seen in Fig. 5B, lanes 5–8, some SOD4 and SOD5 in hyphal extracts is extracted by SDS treatment of membrane and cell wall containing fractions. The same is true for yeast-form *C. albicans*, expressing either endogenous SOD4 (Fig. 5D), or engineered to express SOD5 under control of the SOD4 promoter (Fig. 5C, lanes 2 and 3). As was seen for secreted SODs (Fig. 5A), the SDS-extracted SODs are larger than expected, ~30 kDa (Fig. 5, B–D). Moreover, SDS-extracted SOD5 is reactive with lyticase, consistent with glucan modification (Fig. S4). We thus obtained no conclusive evidence that either SOD4 or SOD5 are membrane localized.

SODs are typically in close proximity to sites of superoxide generation, and we therefore expected to see a prominent membrane localization for SOD5 in hyphae that express membrane FRE8. Instead, our studies show that SOD5 is most prominent in the cell wall of hyphae (Fig. 5B, top), and the same is true of yeast-form cells (Fig. 5C). It is important to note that FRE8 superoxide is confined to a very small component of hyphae, namely the hyphal tip (17). Therefore, only a small fraction of total SOD5 (or SOD4 with Fe-starved hyphae) may be relevant to dismutation of FRE8-derived superoxide. This low level of membrane SOD may escape our detection.

**Dual-regulated Cu-only SODs in other Candida species**

*C. albicans* has evolved to express virtually identical Cu-only SODs under disparate growth conditions of morphogenesis and Fe starvation. Is this true of other fungal species? Like *C. albicans*, the closely related *Candida dubliniensis* has three Cu-only SODs (SOD4, SOD5, and SOD6) with 85–95% identity to their corresponding SODs in *C. albicans* (Fig. S2). However, other *Candida* species such as *Candida tropicalis* and *C. auris* have only two Cu-only SOD candidates: a single SOD similar to *C. albicans* SOD4 and SOD5 (denoted here as CtSOD4 and CauSOD4) and a larger SOD6-like SOD, denoted here as CtSOD6 and CauSOD6.

As with *C. albicans*, *C. tropicalis* is a diplomoid opportunistic fungal pathogen that exists in yeast-form, hyphal and pseudo-hyphal states (60, 61). Yeast-form *C. tropicalis* can be starved of Fe by growth in the presence of BPS (Fig. 6A) and is the case for *C. albicans*, BPS does not induce a Cu-starvation stress state (Fig. S3A). Under Fe starvation induced by BPS, CtSOD4 but not CtSOD6 is strongly induced (Fig. 6B). As such, CtSOD4 appears analogous to Fe-regulated SOD4 of *C. albicans* (Fig. 4C). To test whether any of the two Cu-only SODs are induced during *C. tropicalis* morphogenesis, cells were stimulated to produce elongated hyphae by growth in 50% serum (Fig. 6C). Under these conditions CtSOD4 is induced, but not CtSOD6 (Fig. 6D). Because CtSOD4 is induced in hyphal cells, we tested whether *C. tropicalis* makes ROS during morphogenesis using the luminol chemiluminescence assay (17). The high concentration of serum needed to induce elongated hyphae in Fig. 6C and D, caused interference in the chemiluminescence assay; therefore we utilized serum-free IMDM medium to stimulate morphogenesis. With IMDM, *C. tropicalis* forms germ tubes or short hyphae (Fig. 7A), and the induction of CtSOD4 was not as prominent as seen with elongated hyphae in serum (compare Figs. 7B and 6D). Nevertheless, a burst of ROS could be seen with cells undergoing morphogenesis in IMDM but not in yeast-form cells (Fig. 7C). This ROS was eliminated by the DPI inhibitor of NOX enzymes (Fig. 7D), suggesting that like *C. albicans*, *C. tropicalis* generates ROS during morphogenesis through a fungal NOX.

It is interesting that *C. tropicalis* induces the same Cu-only SOD under Fe-starvation and morphogenesis conditions, whereas *C. albicans* uses separate Cu-only SODs for these two states. *C. albicans* SOD4 and SOD5 are virtually indistinguishable in terms of catalytic activities, Cu co-factor binding properties and cellular localization (Figs. 2, 3, and 5), but they diverge in surface charges outside the active site (Fig. 1B).

**Figure 6. **6. *C. tropicalis* expresses a single Cu-only SOD for Fe starvation and morphogenesis. *C. tropicalis* cultures were grown as either yeast-form cells in the presence or absence of 150 μM BPS (A and B), or induced to form hyphae by growth in 50% serum for 4 h (C and D). Cells were analyzed for (A) Fe content by ICP-MS where results represent the averages of 3–4 independent cultures, *p = 0.0147, or (B) and D) expression of the indicated genes by qRT-PCR relative to *C. tropicalis* TUB2, where results represent averages of 3–5 cultures over 2 experimental trials. *p = 0.0107; **p = 0.005; ***p = 0.0002. There is no statistically significant difference in SOD6 expression in D as determined by unpaired t test. C. cells used for the qRT-PCR of D were analyzed by dark field microscopy. Scale bar = 10 μm.
C. albicans SOD4 and SOD5 may therefore be tailored to interact with different molecules under their cognate Fe-starvation and hyphal-morphogenesis conditions. C. tropicalis SOD4 shares ~60% identity with C. albicans SOD4 and SOD5 (Fig. S2), and the predicted surface charges cannot be matched with either of the two C. albicans SODs. The unique attributes of the C. tropicalis SOD4 polypeptide may bestow the SOD with the capacity to operate under both Fe-starvation and morphogenesis conditions.

C. auris is an emerging fungal pathogen of great public health concern. This yeast lies in the Candida CTG clade, but is more divergent compared with other members including C. albicans, C. dubliniensis, and C. tropicalis (62, 63). Unlike the three aforementioned species, C. auris is haploid and does not readily generate hyphae in response to environmental cues such as serum or elevated temperature (64). Filaments have been reported for C. auris recovered from infected mouse livers, but the mediators of this morphogenesis are unknown and appear distinct from those of other CTG clade species (65). The predicted CauSOD4 of C. auris is ~35% identical to C. albicans SOD4 and SOD5, and given this relatively low sequence homology, we addressed whether CauSOD4 is an actual SOD. We engineered C. albicans to secrete the recombinant CauSOD4 and assayed the extracellular growth medium for SOD activity as was previously done for C. albicans SOD5 (4, 5). As seen in Fig. 8A, lanes 5 and 6, CauSOD4 secreted from C. albicans is active under two culture pH conditions, similar to secreted recombinant C. albicans SOD4 (lanes 3 and 4) and C. albicans SOD5 (5).

We also examined expression of CauSOD4 and CauSOD6 mRNA in laboratory cultures of C. auris. Although these strains cannot form hyphae under laboratory conditions (64), a Fe-starvation state can be induced using BPS (Fig. 8B). As with the other Candida species, there is no evidence for Cu starvation induced by BPS in C. auris (Fig. S3). The Fe-starved C. auris cells remain in the yeast-form state (Fig. 8C). As seen in Fig. 8D, CauSOD4 is induced during Fe starvation, similar to SOD4 of C. tropicalis and C. albicans. CauSOD6 if anything, is somewhat repressed under these conditions (Fig. 8D).

Our results suggest, then, that for the Candida species evaluated here, Fe limitation induces expression of Cu-only SODs. In those species that readily make hyphae, hyphal morphogenesis induces Cu-only SOD activity coincident with the ROS burst of morphogenesis. The link between morphogenic signals and expression of Cu-only SODs extends beyond Candida species. The Pezizomycotina fungus Neurospora crassa (bread mold) uses NOX ROS to signal morphogenesis (19) and has a GPI-anchored cell wall Cu-only SOD (acw-10) transcriptionally induced by hyphal formation (50, 66, 67).

Why are Cu-only SODs induced with Fe starvation?

There is much evidence linking intracellular Fe homeostasis to oxidative stress (68–73), and as previously mentioned, intracellular SODs protect labile Fe-S clusters from oxidative inactivation (2). These Fe-S clusters become even more vulnerable...
under Fe-starvation conditions, increasing the demand for SOD protection. Indeed, intracellular SOD enzymes have been shown to be induced by Fe starvation in the Pezizomycotina fungi *Aspergillus fumigatus* and *Aspergillus nidulans* (74) and the algae *Chlamydomonas reinhardtii* (69), with the intent of guarding Fe-S clusters. However, the rationale for inducing extracellular SODs with Fe starvation is less clear. The fungal Cu-only SODs should protect extracellular/cell-surface targets from damage and to date no extracellular Fe-S enzymes have been identified for fungi. Other targets of oxidative damage must be involved and possibilities include the reductive Fe uptake system of yeasts that may be prone to Fenton chemistry (73, 75) and the plasma membrane itself. In *C. albicans*, the heme protein ERG11 is down-regulated with Fe starvation (76), which increases plasma membrane fluidity through loss of ergosterol, making the fungus more vulnerable to oxidative stress (77). A family of flavoproteins protect the plasma membrane from oxidation of polyunsaturated fatty acids (78), and interestingly, one of these, PST1, is induced by Fe starvation (37). Fe starvation also induces a peroxiredoxin TSA1 (37) that can be secreted (79). Thus, Fe starvation induces a host of antioxidant defenses including Cu-only SODs that may collectively protect membrane integrity during the oxidative attack of the host.

Last, it is possible that induction of Cu-only SODs by Fe-starvation is part of the “adaptive prediction” response of the fungal pathogen. As recently proposed by Brunke and Hube (80), microbes have the ability to predict impending environmental insults based on cues from current environmental conditions. When *Candida* sp. enter the Fe limiting setting of the host, they may pre-adapt to upcoming oxidative insults by inducing antioxidant defenses. Coupling extracellular SOD expression to Fe restriction may help pathogens prepare for the oxidative burst of immune cells in the Fe-starved environment of the host. To our knowledge, our studies are the first to report a connection between extracellular SODs and Fe limitation for any organism and may be more widespread among microbial pathogens.

**Experimental procedures**

**Plasmids, strains, and growth conditions**

Primers used for plasmid engineering are listed in Table S1. The pSS001 plasmid for expressing *C. albicans* SOD4 in *E. coli* was created similarly to that engineered for expressing *C. albicans* SOD5. The expression backbone vector was derived from pAG10H-SOD5 (4), by removing SOD5 coding sequences and linearizing the vector by PCR using primers ORP246R and ORP245F. SOD4 sequences +79 to +543 with respect to the translational start site (encoding SOD4 amino acids 27–181) were amplified using primers OSS01SOD4R and OSS02SOD4F with 15 and 18 base pairs of flanking homology to the aforementioned vector. The amplified sequence was then inserted into the expression backbone vector using the Gibson Assembly Master Mix (New England Biolabs). The pPOJG01 and pMT101 plasmids designed to express and secrete *C. albicans* SOD4 and *C. auris* SOD4 and *C. albicans* were created similarly to that of *C. albicans* SOD5 using plasmid CaEXP, whereby SODs are expressed under the MET3 methionine repressible promoter (4). Sequences encoding residues 1–170 (missing the site for GPI anchorage) of *C. albicans* SOD4 was amplified from SC5314 genomic DNA and inserted into BamHI and PstI sites of CaEXP. For *C. auris* SOD4, sequences encoding amino acids 1–173 (missing predicted sequences for GPI anchorage) were introduced in CaEXP by Gibson assembly using primers OMT01 and OMT02.

All *C. albicans* strains used in this study were derived from clinical isolate SC5314 or KC2 (ura3Δ::imm434/ura3Δ::imm434) (81). Strains containing homozygous deletions in SOD4 and SOD5 as well as strains expressing SOD5 under the SOD4 promoter were generated using the Cas9 system optimized for use in *C. albicans* (82) and oligonucleotides listed in Table S2. *sod5Δ/Δ* and *sod4Δ/Δ* homozygous null mutations were generated using 49-bp segments of donor DNA containing 49-bp homology to the upstream or downstream flanking regions of the gene; guide RNAs directed against either SOD4 or SOD5 were designed using Benchling software (82). The individual *sod5Δ/Δ* and *sod4Δ/Δ* and the double *sod4Δ/Δ sod5Δ/Δ* homozygous null mutations were introduced into SC5314, generating strains SS100, SS101, and SS102, respectively. To place SOD5 coding sequences under control of the SOD4 promoter, a large donor sequence encompassing residues −242 to −1 of SOD4 upstream and +700 to +941 SOD4 downstream sequences flanking SOD5 coding sequences +1 to +687 was used. This SOD4 promoter–SOD5 fusion was introduced in both the *sod4Δ/Δ* SS101 and *sod4Δ/Δ sod5Δ/Δ* SS102 strains using an Add-TAG guide DNA specific to the *sod4Δ/Δ* deletion, generating strains SS103 and SS104, respectively. Strains engineered to secrete recombinant *C. albicans* SOD4 and *C. auris* SOD4 were generated using plasmids pPOJG01 and pMT001 introduced into the RP10 locus of *C. albicans* strain KC2 as precisely described for SOD5 (5). The SN152 and isogenic *sef1Δ/Δ* null strain have been previously published (83) and were obtained from the Fungal Genetics Stock Center. All *C. albicans* strains generated here were verified by PCR and/or gene sequencing. *C. tropicalis* (Castellani) Berkhout 750 strain was purchased from ATCC. Two isolates of *C. auris* were kind gifts of Sean Zhang and correspond to previously published isolates 2 and 6 from the Center of Disease Control (84).

All three yeast species (*C. albicans*, *C. tropicalis*, and *C. auris*) were maintained by growth at 30 °C in a 1% yeast extract, 2% peptone based medium with 2% glucose (YPD). To achieve Fe- or Cu-starvation conditions, YPD medium was supplemented with 150 μM BPS or 800 μM BCS, respectively, and cells grown overnight to mid-log phase, typically OD₆₆₀ 1.0–2.0. In the case of *C. albicans*, elongated hyphae were obtained by growth in IMDM as described (17). Cells were first grown in YPD to mid-log phase, starved in H₂O at 30 °C for 30 min, and then inoculated into pre-warmed IMDM at a starting OD₆₆₀ of 0.1. Hyphae were allowed to form for 2–16 h at 37 °C as indicated. IMDM is not supplemented with additional Fe and the amount of Fe in various lots of this commercial media can vary between ∼25 and ∼100 nM, the former of which can induce an Fe-starvation state in *C. albicans*. For *C. albicans* cells expressing and secreting *C. albicans* SOD4 and *C. auris* SOD4, a synthetic complete media based on yeast nitrogen base and missing methionine and cysteine (SC-Cys-Met) was used as described.
(4, 5). For *C. tropicalis*, elongated hyphae or short germ tube formation was obtained by growth in serum or IMDM, respectively. Cells were first grown in YPD to OD_{600} of ≈20, then starved in H_{2}O at 30 °C for 30 min. For serum induction of hyphae, starved cells were incubated at 37 °C in 50% fetal bovine serum (F6178 from Sigma), 50% H_{2}O for 4 h. For IMDM induction of germ tubes, cells were incubated for 3 h in IMDM under Fe-replete conditions. All fungal cells were photographed using dark field microscopy using a Nikon Infinity 1 microscope at ×40 magnification.

**Purification and biochemical analysis of recombinant SOD4 and SOD5 expressed in E. coli**

Expression, purification, and Cu reconstitution of recombinant *C. albicans* SOD4 and SOD5 was accomplished essentially according to published methods (4, 5). The proteins containing an N-terminal His_{10} tag and intervening TEV protease-cleavage site are not soluble when expressed in *E. coli*, but can be isolated from inclusion bodies and solubilized by incubation in 8 M urea. The totally denatured SOD4 protein was refolded in a Tris/GSH redox buffer at pH 8.0, subjected to nickel affinity chromatography and removal of the His tag by TEV cleavage, followed by Q-Sepharose Fast Flow anion exchange column chromatography (4, 5). Purification of recombinant SOD4 and SOD5 followed identical procedures except during the final anion exchange step, where the bound fraction rather than the flow-through was collected in the case of SOD4 (Fig. 1A).

Reconstitution of SOD4 and SOD5 with Cu and subsequent removal of unbound Cu involved a series of dialysis steps against 4 liters of buffer at 4 °C similar to published procedures (4, 5). Reconstitution was carried out either in acetate buffer, pH 5.5, as previously described, in 25 mM BisTris, pH 6.9, or in 10 mM Tris, pH 8.0. The Cu binding equivalents to SOD4 were determined using an extinction coefficient of 13,075 M^{-1} cm^{-1} for SOD4 protein and Cu measured by atomic absorption spectrophotometry (AAS) on an AAAnalyst 600 Graphite Furnace atomic absorption spectrometer.

**Biochemical analyses of recombinant SOD4 and SOD5 from E. coli**

Analytical ultracentrifugation experiments were carried out essentially as described (4) at the Center for Molecular Biophysics at The Johns Hopkins University. Samples contained 0.75 mg/mL of recombinant SOD4 or SOD5 or an equal mixture of the two in 25 mM BisTris, pH 6.9. Measurements were obtained using interference optics with a laser wavelength of 655 nm.

Cu-binding stability measurements on Cu-bound SOD4 were carried out by equilibrium dialysis against EDTA, glycin, and PAR essentially as described (23). 10 μM Cu-SOD4 in a buffer of 25 mM KPO_{4}, pH 8.0, was subject to dialysis using a minidialysis device (Thermo Fisher Scientific, 88401) against 1 mM EDTA, 1 mM glycin, 0.25 mM PAR or against buffer alone at 25 °C as described (23). At the indicated time points up to 72 h, 10-μL aliquots of the SOD containing solution were analyzed for Cu by AAS. Calculations of stability constants for Cu binding to SOD4 were obtained from results of dialysis against glycin and PAR using equations previously described for SOD5 (23).

Pulse radiolysis experiments were performed at Brookhaven National Laboratory using the 2 MeV Van de Graaff accelerator as previously published (4, 5). Pulses lasting from 100 ns to 1.6 μs allowed generation of 1.8–26 μM superoxide anion, and the rates were determined from an average of 4 or more measurements. Unless indicated otherwise, SOD4 and SOD5 were used at 1–2.6 μM. The decay of superoxide anion was monitored spectrophotometrically at 260 nm, and in the presence of SOD4 or SOD5, the time-dependent absorption profiles were fit to a first-order process using an in-house kinetic fitting program (PRWIN, H. Schwarz, BNL Pulse Radiolysis Program). Reactions were carried out using air-saturated solutions at room temperature (22 °C) containing 20 mM sodium formate, 20 mM potassium phosphate, 2.5 mM Chelex-treated Tris, and 10 μM sodium EDTA. The solution pH was adjusted using NaOH (Baker ultrapure) or H_{2}SO_{4} (Ultrex). Total Cu concentrations were determined by AAS. The Cu concentrations reported here represent SOD4 or SOD5-bound Cu and not free Cu, as corrected by measuring the rate of superoxide decay in the presence and absence of EDTA as described previously (4, 5). These short incubations with EDTA did not affect binding of catalytic Cu, as there was no loss in activity when comparing rates with the first pulse and the last pulse. The effects of ionic strength on SOD4 activity was determined by plotting rate constants as a function of increasing concentrations of NaCl. The log(k_{calc}) versus √[I] was plotted as previously described for SOD5 (4) and Cu/Zn-SOD1 (30–32).

Analysis of SOD enzyme activity by reduction of WST-1 (2-(4-iiodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt) was carried out essentially as described (23). Briefly, recombinant SOD4 and SOD5 in 50 mM KPO_{4}, pH 7.8, were assayed in triplicate 200-μL reactions in a 96-well-plate format containing 0.1 mM xanthine, 0.2 mM EDTA, 0.16 milliunits of xanthine oxidase, and 0.3 mM WST-1 (Dojindo). Following incubation at 37 °C for 45 min, the SOD inhibition of WST-1 reduction by superoxide was measured by absorbance at 450 nm.

**Biochemical analyses of Candida cells in cultures**

For RNA analysis by qRT-PCR, 10 OD_{600} units of cells were washed twice with diethyl pyrocarbonate-treated water, and RNA was isolated using acid phenol/chloroform extraction (43). Cell pellets were resuspended in 400 μL of buffer containing 50 mM NaOAc, pH 5.5, 10 mM EDTA, and 1% SDS. Samples were then extracted twice in phenol, pH 4.5, once in chloroform, followed by ethanol precipitation. Samples were then treated with DNase I and cDNA were prepared using a Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). cDNA was then diluted 1:10 in diethyl pyrocarbonate/H_{2}O and subjected to qRT-PCR using iTaq Universal SYBR Green Supermix (Bio-Rad) and values were normalized to the respective *TUB2* of the *Candida* species using the ΔC_{T} method, where C_{T} is the threshold cycle. Primers used are described in Table S3.
**Fungal Cu-only SODs, ROS, and iron starvation**

For fractionation of *C. albicans* into cell wall, secreted and membrane-containing components, 50-ml cultures were grown as described above for hyphal formation or yeast-form cells. Cells were pelleted and media were collected as secreted fraction. The cell pellets were washed in 2 mM phenylmethylsulfonyl fluoride and protease/phosphatase inhibitor mixture (Cell Signaling Technology). Cells were then subjected to homogenization with 0.5-mm diameter Zirconia/silica beads (Research Products International) using three 1.5 cycles on a Beadblaster benchtop homogenizer (Benchmark). The mixture was then centrifuged at 14,000 \( \times g \) for 10 min at 4 °C. The pellet was washed in lysis buffer to generate the post-lysis cell pellet containing cell wall and membrane fractions. In the case of cell wall preparations, the post-lysis cell pellet was resuspended in 150 \( \mu l \) of lysis buffer containing 30 units/ml of *Arthrobacter luteus* Lyticate (Millipore Sigma) and incubated 3 h at 30 °C. Following centrifugation at 14,000 \( \times g \) for 10 min at 4 °C, the supernatant was collected and clarified by a second centrifugation. The clarified supernatant contained GPI-anchored proteins liberated from the cell wall and represented the cell wall fraction. For membrane fractions, the post-lysis cell pellet was resuspended in 150 \( \mu l \) of lysis buffer containing 2% SDS; samples were boiled for 30 min and submitted twice to centrifugation at 14,000 \( \times g \) for 10 min at room temperature. The clarified supernatant contains proteins liberated by SDS and was designated as the SDS/membrane fraction. For the secreted fraction, the media was filtered through 0.2-\( \mu m \) filters and concentrated 100-fold using a 10,000 Da molecular mass cut-off filter. 60 \( \mu l \) of the secreted, and 30 \( \mu l \) of the cell wall and SDS/membrane fractions were deglycosylated in a 100-\( \mu l \) reaction using PNGase F according to the manufacturer’s specifications (New England Biolabs), except reactions proceeded for 6 h at 37 °C. Where indicated, 30 \( \mu l \) of the secreted or SDS/membrane fractions were treated with 0.1 or 0.5 mg/ml of lyticate, respectively, in 100-\( \mu l \) reactions for 2 h at 30 °C, prior to PNGase treatment as described above. One-third of the reactions were then subjected to immunoblot analysis for SOD4 and SOD5.

For preparation of soluble cell lysates from *C. albicans* and *C. auris*, 10 \( OD_{600} \) units of cells were harvested, washed in water, and resuspended in 150 \( \mu l \) of a lysis buffer containing 5 mM EDTA, 5 mM EGTA, 50 mM NaCl, 10% glycerol, and 0.1% Triton X-100 in 10 mM sodium phosphate, pH 7.8. An equal volume of 0.5-mm zirconium oxide beads (Research Products International) were added and cells were subjected to bead homogenization by two cycles of 2 min vortexing at 4 °C. Samples were clarified by centrifugation for 10 min and the supernatant represented soluble lysates.

Immunoblot analysis was carried out by SDS reducing gel electrophoresis on 4–12% BisTris acrylamide gels (Thermo Fisher) followed by transfer to PVDF membranes and incubation with either anti-SOD4 antibody at a 1:2,500 dilution or anti-SOD5, anti-SOD1, or anti-PGK1 (Acris) all at 1:5,000 dilution followed by secondary goat anti-rabbit IgG Alexa Fluor 680 antibody at 1:10,000 dilution (Thermo Fisher Scientific). Immunoblots were imaged using Odyssey software at 700-nm channel. Anti-SOD4 polyclonal antibody was prepared using purified recombinant SOD4 from *E. coli* and a 90-day rabbit protocol from Cocalico. Polyclonal anti-SOD5 and anti-SOD1 antibodies were as described (5, 43). Analysis of SOD activity by native gel electrophoresis of soluble lysates and nitro blue tetrazolium staining was carried out as previously published (85).

Fe and Cu levels in *Candida* species and in media were measured using inductively coupled plasma MS (ICP-MS). For cell analysis, 10 \( OD_{600} \) cell units were resuspended in 500 \( \mu l \) of 20% nitric acid and digested overnight at 100 °C. The solution was then diluted in 1:10 in MilliQ water to 2% nitric acid and analyzed by ICP-MS on an Agilent 7700X instrument. Values are normalized to cell number. For measuring ROS production by *C. tropicalis*, a luminol chemiluminescence assay was used (17). Cells were grown in YPD to log phase, water starved for 30 min, and grown in prewarmed Fe-replete IMDM for 3 h at 37 °C. 100 \( \mu l \) of the cell solution was added in duplicates or triplicates in a 96-well-plate format to 100 \( \mu l \) of Hanks’ buffered saline solution also containing 0.2 mM luminol (Cyanam Chemicals) and 0.5 units/ml of horseradish peroxidase. Samples were analyzed for luminol chemiluminescence using a BioTek Synergy HT plate reader for 1.5 h at 37 °C as described (17). Results were plotted according to relative luminescence units (RLU) per second.

To measure activity of SODs secreted from *C. albicans* the aforementioned KC2 strains engineered to express and secrete recombinant SOD4 from *C. albicans* or *C. auris*, or KC2 transformed with empty CaEXP vector were seeded at an \( OD_{600} \) of 0.05 in SC-Cys-Met media and grown for 16 h at 30 °C. Cells were harvested and resuspended in fresh SC-Cys-Met media buffered to either pH 3.3 or 7.5 with 60 mM HEPES to test for pH dependence on Cu activation of the secreted enzyme, as was previously done for SOD5 (5). Following incubation for 1 h at 30 °C, cells were removed by centrifugation and the growth medium was concentrated by filtration (4). The concentrated growth media from the equivalent 40 \( A_{600} \) units of *C. albicans* cells was applied to native gel electrophoresis, and activity of the native glycosylated SOD proteins was analyzed by nitro blue tetrazolium staining as previously described (4, 5).

**Computer analyses**

Sequence alignments were generated using Clustal Omega (86). Coloring of alignment files was generated using the color align conservation tool (https://www.bioinformatics.org/sms2/color_align_cons.html)3 with a 60% identity cutoff between sequences required for coloring. Structural models of SOD4 used to generate surface charge maps were generated using MODELLER with the template PBD ID 4N3T. Electrostatics were calculated using PDBPQR and using PROPKA to assign protonation states at pH 8.0 and generate an input file to be used on the APBS prediction server. Structures were visualized using MACYPMOL.

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