The Phylogeny and Active Site Design of Eukaryotic Copper-only Superoxide Dismutases*

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In eukaryotes the bimetallic Cu/Zn superoxide dismutase (SOD) enzymes play important roles in the biology of reactive oxygen species by disproportionating superoxide anion. Recently, we reported that the fungal pathogen Candida albicans expresses a novel copper-only SOD, known as SOD5, that lacks the zinc cofactor and electrostatic loop (ESL) domain of Cu/Zn-SODs for substrate guidance. Despite these abnormalities, C. albicans SOD5 can disproportionate superoxide at rates limited only by diffusion. Here we demonstrate that this curious copper-only SOD occurs throughout the fungal kingdom as well as in phylogenetically distant oomycetes or “pseudofungi” species. It is the only form of extracellular SOD in fungi and oomycetes, in stark contrast to the extracellular Cu/Zn-SODs of plants and animals. Through structural biology and biochemical approaches we demonstrate that these copper-only SODs have evolved with a specialized active site consisting of two highly conserved residues equivalent to SOD5 Glu-110 and Asp-113. The equivalent positions are zinc binding ligands in Cu/Zn-SODs and have evolved in copper-only SODs to control catalysis and copper binding in lieu of zinc and the ESL. Similar to the zinc ion in Cu/Zn-SODs, SOD5 Glu-110 helps orient a key copper-coordinating histidine and extends the pH range of enzyme catalysis. SOD5 Asp-113 connects to the active site in a manner similar to that of the ESL in Cu/Zn-SODs and assists in copper cofactor binding. Copper-only SODs are virulence factors for certain fungal pathogens; thus this unique active site may be a target for future anti-fungal strategies.

The bimetallic Cu/Zn superoxide dismutases (SODs)3 are one of the most widely studied class of enzymes in biology. Since their discovery by McCord and Fridovich (1) nearly 50 years ago, Cu/Zn-SODs are now known to be nearly ubiquitous in nature (2). These enzymes disproportionate superoxide (O2−) anion via a ping-pong reaction mechanism (overviewed in Equations 1 and 2) where 2 O2− equivalents react in a cyclical fashion with oxidized Cu(II) and reduced Cu(I) enzyme to form O2 and H2O2 (2–4). With rates of catalysis approaching diffusion limits (~10⁹ M⁻¹s⁻¹), they are considered “perfect enzymes.” Eukaryotic Cu/Zn-SOD1 is a homodimer found in virtually every intracellular compartment except for the mitochondrial matrix (5–7), and many eukaryotes express a second Cu/Zn-SOD (extracellular-SOD) that is secreted as a dimer of dimers and associates with the extracellular matrix (8–11). Cu/Zn-SODs are potent antioxidants; they guard against superoxide from aerobic metabolism and environmental oxidants, and they are essential for virulence of pathogenic microbes (12, 13). Cu/Zn-SODs also play diverse roles in cell signaling and have many implications in health and disease including angiogenesis, cancer, and neurodegenerative disease (14–18). SOD1 is one of the best characterized copper-containing enzymes due in part to its link in 1993 to the neurodegenerative disease familial amyotrophic lateral sclerosis (19–21).

\[ \text{Cu}^{II} + \text{O}_2^- \xrightarrow{k_1} \text{Cu}^{III} + \text{O}_2 \]  
(Eq. 1)

\[ \text{Cu}^{III} + \text{O}_2^- \xrightarrow{k_2} 2 \text{H}^+ + \text{H}_2\text{O}_2 \]  
(Eq. 2)

For decades, eukaryotic copper-containing SODs were considered to be exclusively of the bimetallic Cu/Zn architecture. However, we recently described a form of copper SOD in the human fungal pathogen Candida albicans that deviates from its bimetallic Cu/Zn kin (22). C. albicans expresses three extracel-

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The atomic coordinates and structure factors (codes 5KBK, 5KBL, and 5KBM) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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Copper-only SODs (SOD4, SOD5, and SOD6) that are predicted to bind copper but not zinc (22). Additionally, these fungal SODs lack most of the residues corresponding to loop VII, the so-called electrostatic loop (ESL) in SOD1. The C. albicans SOD5 structure revealed that due to the absence of loop VII sequences, the copper sites of these SODs are accessible to solvent, unlike the recessed copper cofactor in Cu/Zn-SODs (22). We also found that C. albicans SOD5 is monomeric, unlike the dimeric and tetrameric Cu/Zn-SODs of other eukaryotes. Despite these differences, we observed that C. albicans SOD5 is a robust SOD enzyme that, like Cu/Zn containing SOD1, is capable of catalyzing the disproportion of superoxide at rates approaching diffusion limits (22).

The high catalytic efficiency of SOD5 was surprising based on the absence of zinc ion requirements and ESL sequences. In canonical Cu/Zn-SOD1, zinc plays a structural role through interactions with the ESL/loop VII and with the copper site via a bridging histidine that binds both copper and zinc. The zinc ion secures the redox active copper cofactor and helps promote SOD catalysis over a wide pH range (pH 5–10) (23, 24). The presence of the ESL/loop VII in SOD1 creates a funnel-shaped cavity that leads to the catalytic copper center and is thought to function in substrate guidance (25–27). Additionally, the ESL stabilizes metal cofactor binding through interactions between a conserved aspartic acid residue in the ESL (Asp-124 in Cu/Zn-SOD1) and ligands for copper and zinc binding. How the fungal copper-only SODs achieve catalytic efficiency and stable copper binding without zinc and the ESL/loop VII was unknown.

How unique is C. albicans SOD5? The only other published report of a copper-only SOD has been of mycobacterial SodC (28). However, prokaryotic SodC is distinct from SOD5 in that it harbors a lengthy ESL/loop VII and there are differences in the active site involving the second coordination sphere to the catalytic copper (see “Discussion” for details). We previously reported the existence of SOD5-like SOD sequences in certain Basidiomycota and Ascomycota fungal pathogens, suggesting these SODs evolved to function under low zinc and high copper conditions at the host-pathogen interface (22). The occurrence of copper-only SODs beyond these fungal pathogens has not been previously explored.

Here we report that SOD5-like SODs are more widespread than originally predicted and occur throughout multiple divisions and phyla of the fungal kingdom in both pathogenic and non-pathogenic fungi. SOD5-like SODs are also predicted to occur in oomycetes of the distant heterokont line of eukaryotes, and we discuss a possible evolutionary basis for how copper-only SODs appeared in these far-removed classes of eukaryotes. In every instance, these copper-only SODs are extracellular, whereas the bimetallic Cu/Zn-SODs of fungi are invariably intracellular. Additionally, we provide structural biology and biochemical insight into the specialized active site of eukaryotic copper-only SODs. Using C. albicans SOD5 as a model, we demonstrate that highly conserved residues Glu-110 and Asp-113 are important elements of the active site that maintain pH-independent catalysis and copper-cofactor binding in the family of eukaryotic copper-only SODs.

Results

Eukaryotic Copper-only SODs Are Specific to the Fungal Kingdom and Oomycetes—We define SOD5-like SODs as ≥20–50 kDa proteins harboring a single Greek key β-barrel fold, a copper-binding site, and disulﬁde-forming cysteine residues in positions similar to those found in Cu/Zn-SOD but without the intact zinc-binding site and loop VII/ESL sequences found in Cu/Zn-SOD (22). Based on these criteria, our analysis of currently available genome sequences reveals that SOD5-like SODs are restricted to eukaryotes. Although mycobacterium express a copper-only SOD (28), this prokaryotic SOD contains an extended loop VII/ESL, excluding it from our analysis. The vast majority of eukaryotic SOD5-like SODs lie within the fungal kingdom, with 160 species identified to date. The distribution of fungal SOD5-like SODs is not uniform but appears restricted to certain fungal classes and clades (Fig. 1A). In all cases examined, SOD5-like SODs are predicted to be extracellular, based on signal sequences for protein secretion. They also typically harbor GPI-anchor sequences for covalent attachment at the cell wall or plasma membrane. Based on our analysis, the only form of extracellular SOD in the fungal kingdom is the copper-only SOD. This is in stark contrast to plants and animals that use the Cu/Zn isoform as extracellular SOD (2, 29).

Aside from fungi, the only other class of eukaryotes predicted to express SOD5-like SODs is oomycetes. As with fungi, these all appear extracellular with some predicted to be GPI-anchored to the cell surface. Oomycetes are heterokonts, placed within the Chromista kingdom also harboring photosynthetic microbes and red algae, a line of eukaryotes distantly removed from the fungal kingdom (Fig. 1A) (30, 31). Despite this phylogenetic distance, oomycetes are often characterized as pseudo-fungi based on their filamentous morphology and invasive lifestyle mimicking that of true fungal pathogens, e.g. the potato blight plant pathogen Phytophthora infestans (30, 32, 33). The apparent convergent evolution of SOD5-like SODs in oomycetes and fungi is addressed below (see “Discussion”).

The Highly Conserved Active Site of Fungal and Oomycete Copper-only SODs—By using the large number of available SOD5-like sequences in fungi and oomycetes, we identified key hallmarks of these copper-only SODs. In all cases, SOD5-like SODs are devoid of the same two zinc binding histidine residues of Cu/Zn-SOD1 (i.e. His-71 and His-80). The position corresponding to zinc ligand His-80 is a highly conserved glutamate or glutamine (SOD5 residue Glu-110) in all eukaryotic copper-only SODs examined to date (Fig. 1B and Fig. 2). Interestingly, two of the zinc binding ligands in Cu/Zn-SOD1 are also present in eukaryotic copper-only SODs, including the position equivalent to SOD1 Asp-83 (SOD5 Asp-113) and the position equivalent to SOD1 His-63 (SOD5 His-93) that bridges Cu(II) and zinc in SOD1 and binds copper in SOD5 (Figs. 1B and 2) (22, 34). For the purposes of this study we will refer to SOD5 His-93 and SOD1 His-63 as the “dynamic histidine” as it coordinates Cu(II) but not Cu(I) in the catalytic cycle (22, 34).

Our studies focused on SOD5 Glu-110 and Asp-113 that interact with the copper site. As seen in the structure of Cu(I) SOD5 (22) (Fig. 2B), a side chain oxygen atom of Glu-110 forms
a hydrogen bond with the nonliganding imidazole nitrogen atom of His-93, which helps to orientate His-93 for Cu(II) binding. In this context, Glu-110 plays a role analogous to that of zinc in the related SOD1 enzyme (Fig. 2A). The side chain of SOD5 Asp-113 interacts with copper binding residue His-75 through a water molecule (Fig. 2B), the latter of which is a position analogous to the side-chain carboxylate OD2 atom of SOD1 Asp-124 in the ESL and that forms a hydrogen bond to the nonliganding imidazole nitrogen of the copper ligand His-46 (Fig. 2A). Based on these interactions, could SOD5 Glu-110 and Asp-113 promote copper catalysis and copper binding in lieu of zinc and the ESL? To address this hypothesis, we engineered Glu-110 and Asp-113 mutants of SOD5 and analyzed their copper binding behaviors, three-dimensional structures, rates of enzymatic catalysis, and activities in cultures of live fungi.

**The Role of Glu-110 in Controlling Catalysis at Elevated pH**—E110A and E110Q substitutions were introduced into recombinant *C. albicans* SOD5 and expressed in *Escherichia coli*. The former variant is predicted to result in loss of the second shell H-bonding interaction between the side chains of Glu-110 and copper ligand His-93 for Cu(II) binding. In this context, Glu-110 plays a role analogous to that of zinc in the related SOD1 enzyme (Fig. 2A). The side chain of SOD5 Asp-113 interacts with copper binding residue His-75 through a water molecule (Fig. 2B), the latter of which is a position analogous to the side-chain carboxylate OD2 atom of SOD1 Asp-124 in the ESL and that forms a hydrogen bond to the nonliganding imidazole nitrogen of the copper ligand His-46 (Fig. 2A). Based on these interactions, could SOD5 Glu-110 and Asp-113 promote copper catalysis and copper binding in lieu of zinc and the ESL? To address this hypothesis, we engineered Glu-110 and Asp-113 mutants of SOD5 and analyzed their copper binding behaviors, three-dimensional structures, rates of enzymatic catalysis, and activities in cultures of live fungi.
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FIGURE 2. Structural comparison of the active sites of Cu/Zn-SOD1 versus C. albicans SOD5. A, active site of S. cerevisiae Cu/Zn-SOD1 (PDB code 1JCV). The copper and zinc metal cofactors are blue and gray spheres, respectively. The dynamic histidine His-63 that coordinates both Cu(II) and zinc is orange, and the ESL with conserved Asp-124 that H-bonds to His-46 is indicated in yellow. B, the active site of C. albicans wild type SOD5 (PDB code 4N3T). Copper is a blue sphere, Glu-110 and Asp-113 are pink, and the dynamic histidine His-93 residue is orange. The Asp-113 orientated water network is shown as small red and blue balls where blue represents the water molecule that interacts with the copper site in a fashion analogous to Asp-124 of the Cu/Zn-SOD1 ESL.

LCMS) (see “Experimental Procedures”). We observed that neither E110A nor E110Q substitutions altered the ability of mutant SOD5 to acquire copper (Table 1). Both mutant enzymes were loaded with copper in >90% yield as determined by atomic absorption spectroscopy (AAS) (Table 1). The Cu(I) structures of E110A and E110Q SOD5 were determined at resolutions approaching 1.4 Å (Table 2 and Fig. 3). The overall folds of the variant proteins as well as the geometries of the copper sites were not affected by these substitutions (Fig. 3). Both variants demonstrate the wild type-like pseudotrigonal planar copper coordination with ligands His-75, His-77, and His-153 serving as equatorial ligands at distances ranging between 2.0 and 2.1 Å (Fig. 3). The NE2 atom (i.e. epsilon-nitrogen) of the dynamic His-93 that only binds Cu(II) lies between 3.4 Å and 3.6 Å of the Cu(I) ion in the E110Q and E110A SOD5 structures, respectively, similar to what has been reported for Cu(I) wild type SOD5 and Cu(I) SOD1 (22, 35).

As expected, the aforementioned H-bonding network connecting Asp-113 to the copper site is not perturbed; however, the H-bond interaction to His-93 is lost in the E110A mutant but not in E110Q (Fig. 3). It is noteworthy that the plane of the imidazole ring of copper ligand His-93 is tilted ~60° in the E110A mutant relative to its orientation in the wild type structure (see overlay, Fig. 3).

To examine the potential impact of these substitutions on catalysis, pulse radiolysis was used to obtain second-order rate constants of superoxide disproportionation. The SOD activity profiles of wild type SOD5 and Glu-110 mutant SOD5 are plotted as a function of pH as seen in the black, blue, and red profiles of Fig. 4. At the lowest pH tested (pH 5.0), E110A and E110Q SOD5 behave like wild type, exhibiting diffusion-limited catalytic activity with second-order rate constants of 1.2 × 10⁹ M⁻¹s⁻¹ and 1.4 × 10⁹ M⁻¹s⁻¹, respectively. These values are similar to that observed for Cu/Zn-SOD1 on a per copper basis (23, 25, 26, 36). However, at higher pH values, the Glu-110 variant activity profiles deviate. We previously reported that wild type SOD5 retains high SOD activity up to pH 8.0 as seen in Fig. 4 (22). By comparison, the E110A mutant is strongly inhibited by increasing pH >5.0, and in fact, measurements of E110A activity above pH 7.0 were not possible, as rates of enzyme catalysis approximated that of the non-catalyzed rate of superoxide disproportionation under our conditions. Glutamine substitution at position Glu-110 (i.e. E110Q) by comparison has a milder effect on pH-dependent catalysis (Fig. 4). At neutral pH 7, the respective E110A and E110Q mutants exhibit 6 and 25% of the activity observed at pH 5.0 compared with the 69% SOD activity retained in WT SOD5 at this same pH (Fig. 4).

We previously reported that the inhibition of wild type SOD5 activity at >pH 8.0 is not due to active site copper loss, and activity can be restored upon solution acidification (22). We observed that the same is true with the Glu-110 mutants. After preincubation at pH 7 and 9 for mutants E110A and E110Q respectively, virtually all SOD activity was restored upon lowering pH indicative of no copper loss (Table 3). The profound inhibition of E110A SOD5 activity at elevated pH must reflect changes in catalytic efficiency.

The pH Dependence of Copper-only SODs Reexamined—The impact of Glu-110 substitutions on SOD5 activity prompted us to revisit the diminished pH independence of copper-only SODs. Although wild type SOD5 retains maximal activity up to pH 8.0, Cu/Zn-SODs such as mammalian SOD1, are pH-independent to pH 10 (37), attributed to the zinc ion, as zinc-free (but copper replete) SOD1 activity is alkaline pH-sensitive (23, 38). However, unlike SOD5, the inhibition of zinc-free SOD1 activity at pH > 8.0 was attributed to loss of copper or migration of copper into the empty zinc site (23, 36). The alkaline pH inhibition of SOD5 occurs without copper loss, and we sought to determine the mechanism.

The catalytic cycle of superoxide disproportionation is a two-step reaction, and we examined whether the superoxide oxidation (see Equation 1) or superoxide reduction (Equation 2) step was affected by alkaline pH. Using pulse radiolysis, we probed wild type SOD5 catalysis at elevated pH (i.e. pH 9.5) with defined substrate-to-enzyme ratios approximating single-turnover conditions. With low substrate doses, we can specifically monitor the first half-reaction (Equation 1) of superoxide disproportionation involving the reduction of Cu(II) and oxidation of superoxide (k₄). Under these conditions we observe biphasic kinetic behavior for the loss of superoxide substrate (Fig. 5A), which is dependent on starting enzyme concentration and is not observed in sequential pulses (Fig. 5, B and C). By

TABLE 1
Copper loading of wild type and mutant SOD5
ND, not detectable.

| Enzyme | pH loaded | Cu/SOD5 | S.D.* |
|--------|-----------|---------|-------|
| WT     | 5.5       | 0.89    | ± 0.01|
| E110A  | 5.5       | 0.91    | ± 0.04|
| E110Q  | 5.5       | 0.93    | ± 0.02|
| D113N  | 5.5       | ND      | ND    |
| D113N  | 7.8       | 1.6     | ± 0.01|

* Molar ratio of copper bound to SOD5 protein and S.D. of values obtained as calculated under “Experimental Procedures.” Unlike D113N, wild type, and E110 mutant SOD5 were efficiently loaded with copper at pH 5.5 and did not require copper reconstitution trials at elevated pH values.

D113N has additional copper bound that is not removable by EDTA. See “Experimental Procedures” for more information.
fitting the reaction profiles for the initial pulses with a two exponential model, we obtained a $k_1$ rate constant of $5.4 \times 10^8 \text{m}^{-1}\text{s}^{-1}$ at pH 9.5 (see Fig. 4, black open square) that is significantly faster than the overall catalytic rate $k_{\text{cat}}$ of $5.57 \times 10^7 \text{m}^{-1}\text{s}^{-1}$.

Because the first half-reaction ($k_1$) is near diffusion limits at high pH, the second half-reaction with the reduced Cu(I) enzyme complex (Equation 2; rate = $k_2$) must be the rate-limiting step. As a second method to probe the two half-reactions, we directly monitored redox changes in the SOD5 copper site. C. albicans SOD5 in the oxidized Cu(II) form possesses a characteristic d-d absorbance band at 720 nm ($\epsilon \sim 150 \text{ M}^{-1}\text{cm}^{-1}$) that can be used to measure the reaction rate ($k_2$) of superoxide with the Cu(II) SOD5 enzyme. Fig. 6 displays the spectral change at 720 nm, corresponding to the loss of Cu(II) SOD5 upon the addition of a substoichiometric pulse of superoxide (5 $\mu$M) under high pH conditions (pH 8.0–10.0). The loss of Cu(II) enzyme follows first-order kinetics, and second-order rate constants ($k_2$) for the reaction of Cu(II) with superoxide were obtained. At all elevated pH conditions tested, the individual rate constants ($k_2$) remain near the diffusion limit (see Fig. 5D, solid black squares) and are in good agreement with those obtained by measuring superoxide substrate decay (open square, Fig. 5D). Together these data unequivocally confirm that the pH-sensitive catalytic behavior of SOD5 is due to alkaline pH inhibition of the $k_2$ half-reaction or the reduction of superoxide by Cu(I) (Equation 2).

Having established the basis for alkaline pH effects on wild type SOD5, we tested whether the drastic deviation in pH dependence with Glu-110 mutants was also due to inhibition of the $k_2$ half-reaction. Analysis of superoxide decay at 260 nm was conducted with E110A and E110Q SOD5 at pH 7.0 and 8.0, respectively, where the mutants exhibited an $\sim$40-fold loss in activity, analogous to the impact of pH 9.5 on wild type SOD5. As with wild type SOD5, a biphasic kinetic behavior was observed at near stoichiometric ratios of enzyme:substrate, and the extent of the fast reaction in the initial pulse was dependent on the starting enzyme concentration. Calculated second-order rate constants for the SOD5 active site mutants are represented by red and blue open squares in Fig. 4. As with wild type SOD5, the Cu(II) reduction rate constants ($k_2$) of the Glu-110 mutants remained diffusion-limited. Therefore, the inhibition in $k_{\text{cat}}$ with Glu-110 substitutions is due to inhibition of $k_2$ alone. We conclude that Glu-110 at the active site helps minimize alkaline pH inhibition of the $k_2$ step of catalysis and ensures maximal activity in physiological pH up to 7.5.

The Role of Asp-113 in SOD5 Copper Binding—Asp-113 in SOD5 is invariant among copper-containing SOD enzymes, both copper-only and Cu/Zn isoforms. In Cu/Zn-SODs, this invariant Asp functions as a zinc ligand, but the rationale for conserving this Asp across all eukaryotic copper-only SODs was unknown. To this end we created D113A and D113N variants that were expressed in either the native C. albicans host or in E. coli for large scale purification and biochemical analyses. D113A SOD5 could only be expressed in C. albicans (see text below) but not in E. coli as several attempts to properly refold this mutant failed. D113N SOD5 was faithfully produced in both E. coli and C. albicans systems and was used for biochemical analyses.

The structure of metallated D113N SOD5 expressed in E. coli was determined by x-ray crystallography and refined to $\sim$1.4 Å (Fig. 3 and Table 2). The D113N substitution did not perturb the overall fold of the protein nor the active site, although it demonstrated more flexibility in regions of the so-called “zinc loop” (residues 98–102). The copper-binding site

| PDB code | SOD5 E110A | SOD5 E110Q | SOD5 D113N |
|----------|------------|------------|------------|
| Data collection | P212121 | P212121 | P212121 |
| Space group | a, b, c (Å) | 34.3, 40.6, 101.9 | 34.3, 40.4, 102.3 | 34.5, 39.5, 102.9 |
| Wavelength (Å) | 1.54178 | 1.54178 | 1.54178 |
| Resolution (Å) | 0.80–1.35 | 0.80–1.35 | 0.80–1.35 |
| $R_{	ext{merge}}$ | 0.041 (0.111) | 0.044 (0.394) | 0.044 (0.411) |
| $R_{	ext{free}}$ | 0.017 (0.046) | 0.023 (0.211) | 0.023 (0.213) |
| Completeness (%) | 90.2 (77.9) | 96.9 (99.9) | 97.0 (88.1) |
| Wilson value (Å²) | 34.3, 40.6, 101.9 | 34.3, 40.6, 101.9 | 34.3, 40.6, 101.9 |
| Refinement | | | |
| Resolution (Å) | 28.47–1.41 | 37.53–1.41 | 28.68–1.41 |
| No. reflections | 25,341 | 27,023 | 23,994 |
| $R_{	ext{work}}$, $R_{	ext{free}}$ | 0.154/0.195 | 0.156/0.204 | 0.217/0.247 |
| No. atoms | 1,180 | 1,173 | 1,105 |
| Protein | 38.5 | 50.6 | 45.3 |
| Water | 27.1 | 33.6 | 35.6 |
| Root mean square deviations | | |
| Bond lengths (Å) | 34.3, 40.6, 101.9 | 34.3, 40.6, 101.9 | 34.3, 40.6, 101.9 |
| Bond angles (°) | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 |

*Highest resolution shell is shown in parentheses.
displays similar pseudotrigonol planar geometry with Cu(I) bonding distances to His-75, His-77, and His-153 residues of 2.0–2.1 Å. However, the network of water molecules that connect to His-75 is altered in the D113N variant (Fig. 3).

By pulse radiolysis, D113N is a highly efficient SOD enzyme with catalytic activity at $k_{cat} = 1 \times 10^9 \text{M}^{-1}\text{s}^{-1}$ from pH 5 to pH 7, mirroring that of wild type enzyme (Fig. 4). D113N SOD5 activity appeared slightly more sensitive to alkaline pH inhibition compared with wild type SOD5, but the effects are minimal compared with substitutions at Glu-110. At pH 7.0 the D113N mutant exhibited 62% of the SOD activity seen at pH 5.0, very comparable with the analogous 69% activity retained with WT SOD5. Like wild type SOD5 and Glu-110 mutants, inhibition of $k_{cat}$ at alkaline pH was found to reflect an inhibition in the $k_2$ half-reaction during catalytic turnover (open green square, Fig. 4).

By far the most prominent effect of the D113N substitution was on copper binding. Typically our recombinant SOD5 proteins are reconstituted with copper at pH 5.5, a methodology that insures accurate and efficient loading of copper into cop-

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**FIGURE 3. Structural comparisons of the active sites of wild type versus Glu-110 and Asp-113 mutants of SOD5.** Active site Cu(I) structures of wild type (PDB code 4N3T), E110Q (PDB code 5KBK), E110A (PDB code 5KBC), and D113N SOD5 (PDB code 5KBM). The top two rows show individual structures, and the bottom row shows overlays of wild type and SOD5 mutants. Cu(I) is a blue sphere, and the dynamic His-93, Glu-110/Asp-113, and residues 154–159 are orange, pink, and yellow, respectively. Water molecules in wild type, E110Q, E110A, and D113N SOD5 are red, gray, light blue, and pink balls, respectively. Dashed lines indicate hydrogen bonds; superimposed structures show only hydrogen bonds of the mutants. Wild type SOD5 crystallized with a Tris molecule (blue, top panel).
per-only SODs including \textit{C. albicans} SOD5 and mycobacterium copper-only 
\textit{SodC} and \textit{Cu/Zn-SODs} (22, 28, 39). WT SOD5 and Glu-110 mutants were readily loaded with copper at pH 5.5; therefore, all our structure-function analyses were conducted with proteins reconstituted under these conditions. Surprisingly, however, recombinant D113N SOD5 was incapable of binding and retaining copper at pH 5.5. Nevertheless, D113N could be efficiently loaded with copper at higher pH (\textit{i.e.} 7.8), yielding an active enzyme (Table 1). Thus, all studies of \textit{E. coli} recombinant D113N were carried out with protein reconstituted with copper at the higher pH.

The effects of pH on copper binding may be particularly relevant for an extracellular enzyme such as SOD5. \textit{C. albicans} typically acidifies its extracellular environment in laboratory cultures to pH < 3 in glucose containing media, and inside the animal host the fungus can thrive in environments of very low pH (e.g. pH ~ 2 of the stomach, slightly acid ~4–5 in the vagina) (40–42). Because SOD5 can acquire its copper ion outside of the \textit{C. albicans} cell (22), the pH of the environment could affect the copper binding process. To address the impact of environmental pH and Asp-113 on copper activation of SOD5, we employed the \textit{C. albicans} fungal expression system (22). In this system, SOD5 is engineered to lack the C-terminal GPI anchor that would otherwise covalently attach SOD5 to the cell wall and prohibit recovery of active enzyme. As such, glycosylated SOD5 is secreted as active enzyme into the growth media (22). In the experiment of Fig. 7, we assayed the activity of wild type \textit{versus} Asp-113 SOD5 mutants secreted from \textit{C. albicans} cultured in either acidic (\textit{pH} ~3.0) or near neutral (7.5) pH conditions. Regardless of the pH, wild type SOD5 was capable of acquiring its essential metal cofactor and exhibited robust activity as determined by a native gel activity assay (Fig. 7, \textit{top}). The only effect of pH was on SOD5 mobility, evidently due to changes in protein glycosylation, as this mobility shift was also seen in immunoblots of fully glycosylated (\textit{−EndoH}), but not deglycosylated (\textit{+EndoH}) SOD5 (see Fig. 7 and the legend).

Unlike wild type SOD5, the Asp-113 mutants exhibited a strong dependence on pH for activity. The Asp-113 mutant polypeptides were secreted from the fungus under all conditions tested (\textit{+EndoH} immunoblot, Fig. 7) but were catalytically inactive in cells cultured under low pH. By comparison, both D113A and D113N derivatives displayed robust activity with cells cultured under near neutral pH (Fig. 7). These findings support observations with recombinant protein from \textit{E. coli} whereby D113N can only bind copper under acidic pH conditions (Table 1). We conclude that in SOD5, the invariant Asp, Asp-113, plays a critical role in stable co-factor binding over a range of pH environments encountered by the fungal species.

**Discussion**

This report focuses on a new class of copper containing SOD enzymes for eukaryotes that deviate from classical Cu/Zn-SODs in three-dimensional structure and metal co-factor requirements. These SODs that use a single copper cofactor occur throughout the fungal kingdom and in oomycetes of the heterokont line of eukaryotes. We show here that these extracellular copper-only SODs have been fine-tuned in evolution to function without zinc and an ESL using a specialized active site. Specifically, active site residues equivalent to SOD5 Glu-110/Gln-110 and Asp-113 are hallmarks of this SOD family and function through second sphere coordination to ensure robust catalysis and copper cofactor binding.

It is curious that among eukaryotes, SOD5-like SODs are only found in phylogenetically distant fungi and oomycetes. Fungi share a common ancestor with animals, whereas heterokonts including oomycetes are thought to have evolved from photosynthetic microbes (30). Despite this vast phylogenetic separation, oomycetes and fungal pathogens show remarkable phenotypic similarities in their capacity for substrate invasion through branched hyphae and extensive filamentation. The striking parallels in oomycete and fungal lifestyles were originally attributed to convergent evolution but more recent genomic analyses have identified underlying horizontal gene transfer events (43). Specifically the \textit{Phytophthora} lineage of oomycetes is thought to have acquired genetic material from an ancient fungal ancestor to the \textit{Magnaporthe} and \textit{Aspergillus} lineages of Ascomycota fungi (44). All three classes of organisms are predicted to express copper-only SODs. Thus the appearance of SOD5-like SODs in fungi and oomycetes most
likely reflects the exchange of genetic material between co-habiting fungal and heterokont ancestors.

The positions equivalent to SOD5 Glu-110 and Asp-113 are zinc ligands in the case of Cu/Zn-SODs, and in SOD5 these residues facilitate catalysis and copper binding over a wide pH range. Glu-110 enables high \( k_{\text{cat}} \) to pH 8.0, whereas Asp-113 promotes copper binding from acidic to neutral pH. This pH range for SOD5 is entirely compatible with the lifestyle of *C. albicans* which thrives in host niches that range from highly acidic to near neutral pH (41, 45, 46). Even if exposed transiently to pH > 8.0, SOD5 retains its copper cofactor (ref (22) and Fig. 4) and full SOD activity can be restored once the fungus acidifies its environment (47). The same may hold true for many other fungal and oomycete species that express extracellular SOD5-like SODs.

We demonstrate here that the alkaline pH sensitivity of SOD5 results from an inhibition of the second step of superoxide disproportionation, i.e. the oxidation of the Cu(I) cofactor by superoxide or \( k_2 \) (see Equation 2). Sensitivity to alkaline pH has also been reported for zinc-free versions of Cu/Zn-SOD1 and although much of the inactivation was attributed to a loss in copper binding to the catalytic site (36), at lower pH (5.0 – 8.0), zinc-free SOD1 also exhibits inhibition in \( k_2 \) without changes in copper binding (23). Thus, all zinc-free, copper-containing SODs may be predisposed to inhibition of \( k_2 \) at elevated pH. With Cu/Zn-SODs, the zinc ion guards against these pH effects and with copper-only SOD5, Glu-110 acts to maintain diffusion limited \( k_2 \) up to pH 8.0. In the three-dimensional structure, Glu-110 mirrors the zinc co-factor by interacting with the dynamic His-93 ligand and properly orienting and/or polarizing His-93. Indeed the E110A SOD5 mutant exhibits significant alteration in His-93 orientation (Fig. 3) and a pronounced inhibition of \( k_2 \) at pH > 5.0 (Fig. 4). His-93 orientation (presumably affecting the position of the liganding NE2 atom) could impact \( k_2 \) at neutral pH through mechanisms involving either protonation of the peroxide product and/or formal proton/electron transfer chemistry from the reduced Cu(I) active site to the superoxide substrate.

Position Asp-113 in SOD5 is particularly interesting in that this Asp has been conserved across all copper containing SODs yet connects to the active site through different avenues in Cu/Zn versus copper-only SODs. In Cu/Zn-SODs, this Asp is a zinc ligand whereas in copper-only SOD5, Asp-113 replaces the role of Asp-124 in SOD1 to secure the copper metal cofactor. We observe that Asp-113 interacts with a highly ordered water molecule that in-turn H-bonds with the nonliganding NE2 atom of the His-75 copper ligand; such interactions yield a delta nitrogen histidine-Cu active site configuration identical to that achieved in Cu/Zn-SOD1 using a highly conserved Asp-124 of the ESL (Fig. 2). SOD1 Asp-124 interacts with both copper and zinc binding ligands and as such, Asp-124 mutations lead to copper and/or zinc metal loading defects in SOD1 (48 – 50).
Similarly, substitutions in SOD5 Asp-113 interfere with the ability to bind copper under acidic conditions that are physiological to *C. albicans*. The mechanism for this pH effect is not understood but may involve disruption of the ordered water structure in the area of the active site or de-protonation of the His-75 copper ligand as a pre-requisite for copper coordination.

Mycobacteria such as *Mycobacterium tuberculosis* also express a copper-only SOD variant, however this appears as a distinct class of SOD enzyme. First, bacterial SodC contains loop VII (ESL) sequences and like Cu/Zn-SODs, uses the equivalent of ESL Asp-124 to H-bond to the copper site rather than the equivalent to SOD5 Asp-113 (28). Secondly, the active site of SodC lacks the Glu-110/Gln-110 trademark of eukaryotic copper-only SODs shown here to be critical for catalysis. Instead, orientation of the dynamic histidine residue is fulfilled by the invariant asp (SodC Asp-89 equivalent to SOD5 Asp-113) (28). Mycobacterial SodC may be a hybrid between the Cu/Zn- and eukaryotic copper-only SOD families.

A key remaining question concerns the evolution of extracellular SODs for eukaryotes. Why have fungi and oomycetes chosen the copper-only SOD for their extracellular SOD, whereas other eukaryotes including plants and animals secrete Cu/Zn-SOD? As one possibility, metal ion availability is the driving force for SOD enzyme evolution. Although the Cu/Zn-SODs of mammals have been shown to acquire their metal co-factors in the secretory pathway and arrive at the cell surface in a fully active form (51), the same may not be true for fungal and oomy-

**FIGURE 6. Reduction of Cu(II) SOD5 at elevated pH.** Time-dependent loss of oxidized Cu(II) SOD5 as monitored by change in absorbance at 720 nm at elevated pH upon the substoichiometric pulse of superoxide substrate. The pseudo-first-order fit to the decay for each spectral time course is indicated by the solid red line.

**FIGURE 7. Expression of wild type and Asp-113 mutant SOD5 in *C. albicans* cultures.** *C. albicans* secreting either wild type or the indicated mutant alleles of SOD5 were cultured for 1 h at the designated pH. The growth medium containing freshly secreted SOD5 was concentrated and analyzed for: SOD5 activity by the native gel assay (no EndoH treatment) (top) and SOD5 protein levels by immunoblot in samples treated with EndoH with molecular weight makers indicated on the left (middle). Quantification of this immunoblot reveals expression levels relative to wild type SOD5 at pH 3.3 = 1.0; left, D113N pH 3.3 = 0.75 and wild type pH 7.5 = 0.76; D113N pH 7.5 = 1.2; right, D113A pH 3.3 = 0.65; wild type pH 7.5 = 0.79; D113N pH 7.5 = 0.54. Bottom, effects of pH on SOD5 glycosylation by immunoblot of samples not treated with EndoH with molecular weight makers indicated on the left. Glycosylated SOD5 exhibits more rapid mobility from cells cultured at pH 7.5, indicating that the change in mobility on the native gel (top) reflects alterations in SOD5 glycosylation. The very strong intensity of D113N, pH 7.5, without EndoH presumably reflects the impact of D113N SOD5 glycosylation on the immunoblot (e.g. antibody recognition), as it is not seen in the deglycosylated + EndoH sample.

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The Family of Eukaryotic Copper-only SODs

cette copper-only SODs. We previously demonstrated that C. albicans SOD5 does not rely on the Golgi copper-ATPase for copper loading but is delivered to the fungal cell wall in an apo-
catalytically inactive form that is loaded with copper out-
side the cell (22). It is possible that extracellular zinc is not
sufficiently bioavailable for ready capture by an extracellular
SOD. Alternatively, the absence of a zinc site might preclude
mis-metallation events outside the cell, for example, copper
entering the zinc site as has been shown for Cu/Zn-SOD
enzymes (36, 38, 52). The copper-only SODs are widespread
throughout fungal and oomycete plant and animal pathogens
and in two fungal pathogens have been shown to be important
for virulence (53, 54). The unique open access active site of
copper-only SODs may ultimately prove to be a viable anti-
microbial target for these pathogens.

Experimental Procedures

Database Searches—The presence of candidate copper-only
SODs genes were first identified using cytoscape 3.3.0 using
networks constructed using the EFI-Enzyme Similarity Tool.
The network was initially constructed using the interpro Cu/Zn
binding domain identifier IPR001424 with a 200–500 amino
acid length filter and an initial alignment score of 50. The
resulting network consisted of 2,804 filtered sequences consist-
ing of 141,772 edges. ClustalW was used to align sequences in
individual networks. Copper-only SODs were selected based on
possession of a single Greek-key β barrel SOD-fold domain, the
retention of four copper-binding residues, and two disulfide-
forming cysteines but lacking two zinc binding histidines in the
so-called zinc loop and containing deletions in the ESL or loop
VII.

Expression of Recombinant Mutant and Wild Type SOD5 in
E. coli—Recombinant wild type and mutant SOD5 proteins
were produced in E. coli using expression plasmids encoding for
C. albicans SOD5 (residues 27–181) with an N-terminal
10× His tag and an intervening tobacco etch virus (TEV) pro-
tease cleavage site. Desired active site mutants were created by
primer-assisted site-directed mutagenesis using the pAG10H-
SOD5 plasmid as template (22). The isolation of wild type and
mutant SOD5 protein were achieved as previously described
with minor modifications (22). Briefly, SOD5 proteins were iso-
lated as inclusion bodies and refolded using a Tris/glutathione
reductase buffer, pH 8.0. The insoluble (misfolded) fraction was
removed by centrifugation at 75,000 g and 0.45 μm filtration.
Properly folded SOD5 was then purified using a 5-ml Histrap
HP (GE Healthcare) and eluted with a linear imidazole gradient
(0–0.5 M). The pooled SOD5 fractions were subjected to dial-
ysis against 50 mM Tris, 50 mM NaCl buffer, pH 8, and the
N-terminal His tag was removed by TEV protease. Residual
His-tagged SOD5, free tag, and TEV protease was removed via
reverse nickel ion affinity chromatography. The resulting flow-
through was then passed through a Hi-trap Q column (GE
Healthcare) to remove any aggregates, and resulting SOD5 pro-
tein fractions were used for copper loading experiments.

Copper loading of SOD5 (100-ml samples) was achieved throu-
gh a series of dialysis steps, all conducted in 4 liters of 25
mM sodium acetate buffer at 4 °C and for 8 h unless otherwise
noted. First, proteins were made apo by acidification through
successive dialysis against buffer pH 5.5 and buffer pH 3.8 fol-
lowed by dialysis overnight against the same pH 3.8 buffer con-
taining 10 mM EDTA. The EDTA was then removed by succe-
sive dialysis against pH 3.8 buffer followed by pH 5.5 buffer,
both made metal-free by treatment with Chelex 100 resin (Bio-
Rad). Copper loading occurred through overnight dialysis
against pH 5.5 buffer containing 0.25 mM CuSO₄ followed by
removal of unbound copper by two rounds of dialysis against
pH 5.5 buffer (no copper added). An additional round of dialysis
with 10 mM Tris, pH 8.0, was performed on wild type SOD5
samples used for studies of Cu(II) binding at 720 nm where high
enzyme concentrations were required. The D113N SOD5
mutant protein was unable to be successfully loaded with cop-
per at pH 5.5 as determined by AAS. However, this enzyme was
effectively copper-loaded at higher pH using 25 mM Tris acetate
buffer, pH 7.8, for copper loading and subsequent dialysis to
remove unbound Cu.

All copper-loaded enzyme solutions were filtered and con-
centrated to ~150 μM using 10,000 M₉ spin filters followed by
flash-freezing in liquid nitrogen. Wild type and mutant SOD5
protein purity and identity were verified using qTOF-LCMS,
concentrations calculated using ε = 9065 m⁻¹ cm⁻¹. The sto-
ichiometry of copper bound to SOD5 was determined by copper
AAS, where values represent the average of two independent
measurements. The same batches of SOD5 protein were
employed for both pulse radiolysis measurements and crystal-
lization trials. For all samples, the total copper binding stoichi-
ometry is based on molar equivalents minus that of free copper
concentrations. Free copper concentrations were determined
by pulse radiolysis measurements of total SOD activity of
enzyme solution in the presence and absence of 10 μM EDTA
metal chelator pH 6.0. At this pH, free copper reacts faster with
superoxide than that of the enzyme and can be discriminated
based on the aforementioned EDTA chelation studies. Note:
the D113N mutant sample contained an ~0.7 equivalents
excess copper as determined by copper AAS that was bound to
protein, not free, based on the definition above. The additional
copper must bind to a region in D113N SOD5 that is distinct
from the active site and does not affect enzyme catalysis. Struc-
tural analysis did not reveal the presence of an additional cop-
per metal-binding site within the D113N mutant structure.
Thus, the identity of the excess copper is unknown.

Crystallization, Structure Determination, and Refinement—
Crystals of the SOD5 mutants were prepared as described pre-
viously (22). Data were collected at the University of Texas
Health Science Center, San Antonio x-ray Crystallography
Core Laboratory using a home source Rigaku MicroMax
007-HF equipped with RAXIS-HTC detectors and integrated
and scaled using XDS (55). The crystals of each mutant were
isomorphous with SOD5 Protein Data Bank entry 4N3T (22) so
a rigid body refinement step was applied before manual model
building. Coordinates for all models were refined using
PHENIX (56), including simulated annealing and anisotropic
B-factor refinement, and alternated with manual rebuilding
using COOT (57). Data collection and refinement statistics are
shown in Table 2. The figures were generated using PyMOL
(58).
SOD5 Expressed in Cultures of C. albicans—The previously described pJG120 plasmid that drives expression of C. albicans SOD5 under the MET3 promoter (22) was used to create D113N and D113A SOD5 mutant derivatives by site-directed mutagenesis. The resultant SOD5 mutant alleles were integrated into the genome of C. albicans strain KC2 (ura3Δ::imm434/ura3Δ::imm434) as previously described (22). The recombinant SOD5 used in these studies (residues 1–170) lacks the GPI anchor for cell wall attachment, and enzymatically active SOD5 can be recovered in the growth medium for analysis (22). Cells expressing recombinant SOD were grown to late log phase in synthetic complete media lacking methionine and cysteine (SC-Cys-Met) to induce the MET3 promoter driving SOD5. A total of 200 – 400 A600 units of cells were harvested and resuspended in 25 ml of fresh pre-warmed SC-Cys-Met media that was buffered to either pH 3.3 or pH 7.5 with 60 mM HEPES. After incubation at 30 °C for 1 h, cells were removed by centrifugation, and the growth medium containing freshly secreted SOD5 was concentrated by filtration as previously described (22). Activity of wild type and mutant SOD5 was analyzed by native gel electrophoresis and nitro blue tetrazolium staining as previously described (22) using growth medium from the equivalent of 100 – 150 A600 units of C. albicans cells. Immunoblot analysis of wild type and mutant SOD5 was achieved with ~25 A600 unit eq of concentrated media that was either treated overnight with EndoH endoglycosidase (New England BioLabs) at 37 °C as described (22) or was not treated with EndoH, to visualize the impact of pH on SOD5 glycosylation. Immunoblots were visualized using an Odyssey IR imaging system (Li-Cor Biosystems) employing anti-SOD5 rabbit serum (JH929; 1:5000 dilution) and an Alexa Fluor 680 goat anti-rabbit secondary antibody. Image studio was used to quantify signal intensities of the EndoH–treated samples. The anti-SOD5 rabbit serum was created using purified recombinant E. coli SOD5 protein as described above and a 90-day rabbit protocol (Cocalico Biological, Inc).

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