

Eukaryotic copper-only superoxide dismutases (SODs): A new class of SOD enzymes and SOD-like protein domains

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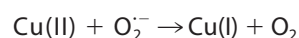
The copper-containing superoxide dismutases (SODs) represent a large family of enzymes that participate in the metabolism of reactive oxygen species by disproportionating superoxide anion radical to oxygen and hydrogen peroxide. Catalysis is driven by the redox-active copper ion, and in most cases, SODs also harbor a zinc at the active site that enhances copper catalysis and stabilizes the protein. Such bimetallic Cu,Zn-SODs are widespread, from the periplasm of bacteria to virtually every organelle in the human cell. However, a new class of copper-containing SODs has recently emerged that function without zinc. These copper-only enzymes serve as extracellular SODs in specific bacteria (*i.e.* Mycobacteria), throughout the fungal kingdom, and in the fungus-like oomycetes. The eukaryotic copper-only SODs are particularly unique in that they lack an electrostatic loop for substrate guidance and have an unusual open-access copper site, yet they can still react with superoxide at rates limited only by diffusion. Copper-only SOD sequences similar to those seen in fungi and oomycetes are also found in the animal kingdom, but rather than single-domain enzymes, they appear as tandem repeats in large polypeptides we refer to as CSRPs (copper-only SOD-repeat proteins). Here, we compare and contrast the Cu,Zn *versus* copper-only SODs and discuss the evolution of copper-only SOD protein domains in animals and fungi.

Superoxide dismutase (SOD)² was discovered in 1969 by McCord and Fridovich (1) as a copper metalloprotein present in bovine erythrocytes that can disproportionate superoxide anion radicals with incredible catalytic efficiency (two-step reaction below).

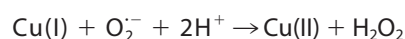
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² The abbreviations used are: SOD, superoxide dismutase; ESL, electrostatic loop; ALS, amyotrophic lateral sclerosis; MtSodC, *M. tuberculosis* copper-only superoxide dismutase C; CSRP, copper-SOD repeat protein; ecSOD, extracellular Cu,Zn-SOD; NOX, NADPH oxidase; GPI, glycosylphosphatidylinositol.



STEP 1



STEP 2

Not long afterward, zinc was also detected in this cuproprotein, establishing mammalian SOD1 as a copper and zinc bimetalloenzyme (2). Since the discovery of Cu,Zn-SODs, distinct classes of SODs that use iron, manganese, or nickel as co-factors have been identified. These SODs are unrelated to Cu,Zn-SODs in primary sequence and structure but share in common the use of a redox-active metal co-factor to disproportionate superoxide (3). SODs protect cells from oxidative stress, particularly in the removal of superoxide produced during metabolism (4, 5), and also have key roles in cell signaling through the local production of H₂O₂ (6–8). In addition, many SODs are virulence factors for pathogens, allowing them to survive the oxidative burst of macrophages and neutrophils at the host–pathogen interface (9, 10).

Cu,Zn-SODs are the only SODs known to function as bimetalloenzymes, requiring copper for catalysis and zinc to enhance catalytic efficiency and stabilize the protein (11–14). For many decades, all members of this SOD family from bacteria to humans were believed to require both copper and zinc. This dogma was challenged first in 2004 with the discovery of a mycobacterial copper-only SOD (15), and then in 2014 with the identification of a large family of copper-only SODs in fungi that not only lacked zinc but contained an unusually open-access copper site (16). Very recent bioinformatics analyses have revealed that copper-only SOD-like protein sequences also occur as repeated protein domains in large molecules we call CSRP (copper-SOD-repeat protein). In this review, we shall compare and contrast the copper-only *versus* Cu,Zn-SOD proteins and discuss the utility of the copper-only SOD protein domain in biology.

Ubiquitous Cu,Zn-SODs

The bimetallic copper- and zinc-containing SODs are widely dispersed in biology from bacteria to mammals and are found in both intracellular and extracellular locations. Virtually all

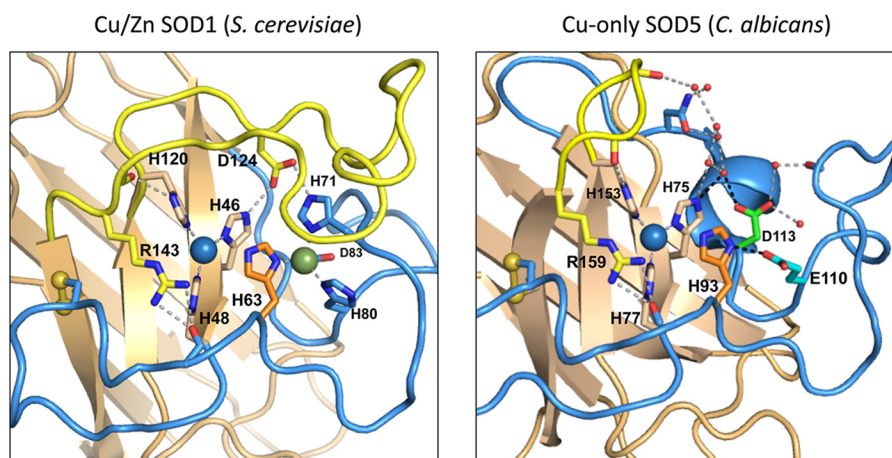


Figure 1. Active site of Cu,Zn versus copper-only SODs. Left and right, comparison of *Saccharomyces cerevisiae* Cu,Zn-SOD1 (left) and *C. albicans* copper-only SOD5 (right) active site with key features highlighted as follows: Greek key β -barrel core (tan), ESL (yellow) with SOD1 Asp-124, active-site arginine (SOD1 Arg-143 and SOD5 Arg-159), disulfide loop (blue) with cysteines as yellow spheres, copper ion (blue), and zinc ion (green) with coordinating residues labeled by number. The dynamic histidine (SOD1 His-63 and SOD5 His-93) is orange, and SOD5 Glu-110 and Asp-113 are cyan and green. Dotted lines represent hydrogen bond networks and small red spheres represent water molecules.

eukaryotes express an abundant intracellular Cu,Zn-SOD typically known as SOD1 (1–3, 17–19). This ubiquitous enzyme is found in various intracellular compartments, primarily in the cytosol (19) but also in the mitochondrial inter-membrane space (20–23), the secretory pathway (24, 25), and even the nucleus (26, 27). SOD1 protects against oxidative damage from metabolic sources of superoxide, including that from the mitochondrial respiratory chain, and also functions in cell signaling involving its H_2O_2 product and peroxide-sensitive kinases and phosphatases (6, 7). In the nucleus, SOD1 can participate in controlling gene expression as has been shown in baker's yeast with gene responses to DNA damage and copper starvation stress (26, 27). SOD1 is a highly abundant protein, and in humans, mutant versions of SOD1 have been linked to an inherited form of amyotrophic lateral sclerosis (ALS). SOD1 misfolding has been implicated in ALS disease, and many excellent reviews have been written on this topic (28–32).

Cu,Zn-SODs can also be found in extracellular locations, and because superoxide does not generally cross biological membranes, the substrate for the extracellular SOD must originate outside the cell. Certain bacteria express Cu,Zn-SODs in their periplasmic space (33–35), and in the case of pathogenic bacteria, these SODs protect against the oxidative burst of the host immune system (9, 10). Many eukaryotes also express a Cu,Zn-SOD distinct from SOD1 that is extracellular. This so-called ecSOD was first discovered by Marklund in 1982 (36) and is a secreted tetrameric protein in extracellular fluid or anchored to the extracellular matrix (3, 36–41). The superoxide substrate for ecSOD is derived from NADPH oxidase (NOX) enzymes (42) that are flavin- and heme-dependent transmembrane enzymes that reduce oxygen to superoxide (42–44). Together, ecSOD and NOX can function in signaling involving reactive oxygen and reactive nitrogen species (8).

Cu,Zn-SODs can be homodimeric (SOD1), tetrameric (ecSOD), or in rare cases monomeric (*Escherichia coli* SodC) (3, 45). Each monomer has several landmark features as follows: a Greek key β -barrel fold; highly conserved copper- and zinc-binding residues; a conserved disulfide; active-site arginine; and

an extended loop VII, also known in eukaryotic Cu,Zn-SODs as the electrostatic loop (ESL) (46). The catalytic copper in the oxidized Cu(II) state is coordinated in a distorted square planar geometry to an axial water molecule and four histidines, one of which (His-63 in the case of yeast and human SOD1) also coordinates zinc (Fig. 1). For the purpose of this review, we shall refer to this bridging His-63 as the “dynamic” histidine based on its on-and-off coordination to copper during catalysis. As superoxide is oxidized in the first step of catalysis, the dynamic bridge between His-63 and copper is broken as Cu(II) is reduced to Cu(I) and detached from His-63, resulting in a trigonal planar geometry for Cu(I). The zinc co-factor remains bound to the dynamic His-63 during catalysis and is additionally coordinated to two other histidines and an aspartate (Fig. 1) (46–48). Although zinc does not directly interact with the superoxide substrate during catalysis, its coordination with the dynamic His-63 assists in the re-oxidation of Cu(I) to Cu(II) in the second step of catalysis and accounts for the large pH independence of SOD activity (11, 12, 14, 49). The zinc co-factor is also important for stabilizing protein structure (13, 14, 50). Additional invariant features of Cu,Zn-SODs include an intermolecular disulfide (48) and an active-site arginine (Arg-143 in SOD1) positioned at the end of the ESL, which attracts and stabilizes the anionic superoxide substrate over the copper-metal center (Fig. 1) (51). With many charged amino acids residing in the ESL, it is believed to create an electrostatic network to funnel the highly solvated superoxide into the active site and accounts for the remarkably rapid rates of superoxide disproportionation (37, 51–54). The ESL is also believed to play a role in stabilizing copper and zinc binding through a network (or series) of hydrogen bonds (11, 50).

Bacterial copper-SOD functions without zinc

For 35 years following the discovery of Cu,Zn-SODs, all copper-containing SOD enzymes were thought to require zinc. However, in 2004 the copper-containing SodC from *Mycobacterium tuberculosis* (MtSodC) was reported to lack zinc and to function with only a single copper atom (15). This copper-only

SOD retains the Greek key β -barrel backbone of Cu,Zn-SODs as well as the same copper-coordination site, disulfide, active-site arginine, and extended loop VII covering the active site (equivalent to the ESL in eukaryotic Cu,Zn-SODs). However, MtSodC is missing the two non-dynamic histidines needed to bind zinc, with one substituted with an alanine and one missing due to a seven-amino acid deletion in the zinc loop (15). Structural analyses indicate zinc is missing from the active site of MtSodC (15).

As mentioned above, the zinc co-factor in Cu,Zn-SODs promotes pH independence in the enzyme and assists in SOD folding/stability. As would be expected for a zinc-less SOD, MtSodC demonstrates diffusion-limited catalysis from pH 6.0 to 8.0, but catalytic efficiency rapidly decreases above pH 8.0 (15). Because *M. tuberculosis* can exist in environments of low pH, e.g. the macrophage phagolysosome (55), this sensitivity to alkaline pH is likely a non-issue. To circumvent the requirement for zinc in protein stability, MtSodC shows an altered dimer interface with a long and rigid loop that is thought to stabilize the protein (15). Copper-only SodC is the only periplasmic SOD in *M. tuberculosis* and has been shown to protect the pathogen from the superoxide bursts of NOX enzymes in activated macrophages (56).

Of note, many SodC sequences in the *Mycobacterium* genus appear to lack the same zinc-binding histidines, indicating that diverse species of *Mycobacterium* SodCs are copper-only (15). Even so, within the eubacterial kingdom, copper-only SOD enzymes appear unique to the *Mycobacterium* genus, as all other periplasmic SODs characterized to date have both copper and zinc (9, 34, 45, 57).

Copper-only SOD in a fungal pathogen

Fungal pathogens, like bacteria, utilize their extracellular SODs as a first line of defense against superoxide generated by macrophage and neutrophil NOX enzymes (9). One of the best studied cases is the extracellular SOD5 from the opportunistic fungal pathogen, *Candida albicans* (58). *C. albicans* expresses three extracellular SODs (SOD4–6) that are members of the Cu,Zn-SOD family and are linked to the cell wall through GPI anchors (59). Of these, SOD5 is the most abundantly expressed in numerous models of candidiasis (60–65) and contributes to virulence in a mouse model of disseminated candidiasis (58). The enzyme is induced during the transition to the hyphal filamentous form required for host invasion (58, 66) and protects *C. albicans* from the superoxide burst of macrophages and neutrophils (63, 67–69). For many years, SOD5 was described as a bimetallic Cu,Zn-SOD (58, 66).

In 2014, three-dimensional structure and biochemical analyses of *C. albicans* SOD5 revealed that it is not a canonical Cu,Zn-SOD (16). Although SOD5 shares the overall Greek key β -barrel fold of Cu,Zn-SODs and exhibits a similar copper-binding geometry, it is missing the same two zinc-binding histidines as *M. tuberculosis* SodC, although in this case both due to amino acid substitutions, not deletions. Furthermore, SOD5 is missing an extensive portion of the ESL/loop VII (Fig. 1) (16). The absence of ESL sequences creates a uniquely open active site where copper is much more accessible to solvent. Attempts to load SOD5 with zinc were unsuccessful, demonstrating that

SOD5 functions with a single atom of copper and no other metals (16). Despite such striking deviations from canonical Cu,Zn-SODs, copper-only SOD5 is an extremely efficient SOD enzyme capable of disproportionating superoxide at maximum rates approaching diffusion limits, $1.8 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ at pH 6.0 (16, 70).

How does copper-only SOD5 function in the absence of zinc and the ESL? As mentioned above, zinc promotes pH-independent catalysis of Cu,Zn-SODs through interactions with the dynamic histidine that also binds Cu(II) (11, 12, 14, 49). In lieu of zinc, the dynamic His-93 of copper-only SOD5 interacts with a conserved glutamate (Glu-110) in the active site (Fig. 1). Disruption of this interaction through Glu-110 mutations alters the orientation of the dynamic His-93 and dramatically decreases the pH range of activity (70). Thus, SOD5 Glu-110 appears to act analogous to zinc, interacting with and correctly orienting the dynamic His-93 to promote rapid catalysis up to pH 8 (70).

The absence of the ESL is perhaps the most striking feature of fungal copper-only SODs, as this highly charged loop was previously reported to be critical for substrate guidance and efficient catalysis in Cu,Zn-SODs (51–54). Despite no ESL, SOD5 shows a strong catalytic dependence on ionic strength, indicative of an alternative form of electrostatic substrate guidance (16). In Cu,Zn-SOD1, the ESL also helps stabilize the copper site through interactions involving ESL Asp-124 and copper coordinating His-46 (Fig. 1, left) (11, 50). In copper-only SOD5, the equivalent copper coordinating His-75 interacts with SOD5 Asp-113 through a hydrogen bond network (Fig. 1, right). Evidence indicates that Asp-113 in the SOD5 active site helps circumvent the need for the ESL in stabilizing copper binding (70). Interestingly, Asp-113 is invariant among all copper-containing SODs reported to date; in Cu,Zn-SODs, this aspartate is a zinc ligand and in copper-only SODs the aspartate functions through interactions with the copper site. This re-purposing of Asp-113 together with Glu-110 represents novel adaptations in the active site of eukaryotic copper-only SODs.

Of note, the features of Glu-110 and Asp-113 described above for fungal SOD5 may not extend to prokaryotic copper-only SODs. *Mycobacterium* SodC retains loop VII/ESL sequences and shows similar loop VII–copper site interactions as seen with Cu,Zn-SOD1 (15). There is no equivalent to SOD5 Glu-110 in mycobacterial SodC; instead, the invariant aspartate (equivalent to SOD5 Asp-113) interacts with the dynamic histidine (15, 70). MtSodC appears to be a hybrid of Cu,Zn and fungal copper-only SODs.

What is the advantage of expressing a copper-only SOD versus a bimetallic Cu,Zn-SOD? Clues may be obtained from examining the metallation process. In mammals, the extracellular Cu,Zn-SOD acquires copper and zinc in the secretory pathway and arrives at the cell surface in an enzymatically active form (71, 72). By contrast, characterization of *C. albicans* SOD5 indicated that the apoprotein is secreted without copper and is only activated upon scavenging copper from the extracellular environment (16). The open active site may help promote such capture of copper outside the cell. With no zinc site, mis-metallation events, such as copper migrating to the zinc site as is seen with Cu,Zn-SODs (49), are obviated. Additionally,

the copper site of *C. albicans* SOD5 appears refractory to mis-metallation by non-native metals such as zinc (16). As such, enzyme activity remains intact regardless of fluctuations in environmental zinc. The copper-only design may indeed be advantageous to controlling enzyme maturation outside the cell.

Role of copper-only SODs in fungal pathogenesis and signaling

Copper-only SODs are widely distributed throughout the fungal kingdom and, in all cases examined thus far, are predicted to be extracellular and attached to the cell wall through GPI anchors (70). Like *C. albicans* SOD5, all fungal extracellular SODs lack zinc binding and ESL sequences and retain the equivalents to Glu/Gln-110 and Asp-113 at the active site. Copper-only SODs are found in many fungal pathogens where they combat the oxidative burst of the host and promote virulence, as has been seen with *C. albicans*, the pulmonary pathogen *Histoplasma capsulatum* (73), and systemic mycosis pathogen *Paracoccidioides brasiliensis* (74). Because of their extracellular location, fungal copper-only SODs may be uniquely positioned with their open active site to acquire copper from the host (16). Curiously, copper-only SODs are also found in non-pathogenic fungi that are not subject to host oxidative attacks, such as the *Tuber melanosporum* truffles fungus (70). With these non-pathogens, the SODs may react with superoxide derived from the fungus itself, similar to how copper- and zinc-containing ecSODs partner with NOX enzymes in mammals as part of signaling through reactive oxygen and reactive nitrogen species (see above). Multicellular fungi are indeed known to use NOX enzymes to signal differentiation (75–77).

Unlike multicellular organisms, unicellular microbes are not generally thought to use NOX enzymes and extracellular SODs for signaling. However, we recently found that copper-only SOD5 from unicellular *C. albicans* can act in signaling involving ROS and a fungal NOX enzyme known as FRE8 (78). *C. albicans* FRE8 and SOD5 together generate H₂O₂ that can help drive morphogenesis of the fungus into an invasive filamentous state (78). Therefore, SOD5 can react with superoxide generated from either the host or the fungal pathogen itself. During infection, one can envision a “superoxide superstorm,” with ROS coming from both the host and the fungal sides of the infection battleground and SOD5 operating at the interface (Fig. 2).

SOD5-like protein domains in animals

We have searched for SOD5-like proteins outside of the fungal kingdom. Our definition of a SOD5-like protein is one with the predicted Greek key β -barrel fold of the Cu,Zn-SOD family, including the copper site, disulfide, and active-site arginine but lacking sequences for zinc binding and the ESL and retaining SOD5 equivalents to Glu-110 and Asp-113. The only non-fungal organisms that express ≈ 20 –30-kDa (predicted molecular mass of mature protein) SOD5-like SODs are oomycetes, a line of heterokont eukaryotes distantly removed from the fungal kingdom (Fig. 3A). As with fungi, the oomycete proteins are predicted to be secreted GPI-anchored extracellular SODs (70). Oomycetes are derived from photosynthetic microbes and are

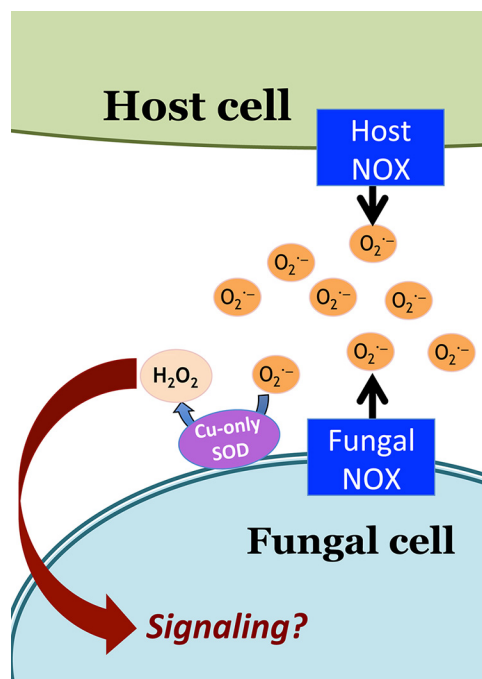


Figure 2. Copper-only SODs can react with superoxide from both the host and fungal pathogen. Shown is a model depicting a fungal copper-only SOD at the host–pathogen interface where it can react with superoxide from host NOX (macrophages or neutrophils) or from fungal NOX. The H₂O₂ generated may be used in signaling as has been shown for *C. albicans* where copper-only SOD5 and the FRE8 NOX promote morphogenesis of the fungus (78).

thought have acquired genetic material through horizontal gene transfer from a fungal ancestor (79–81). Copper-only SODs were apparently carried over as part of this genetic transfer.

Interestingly, SOD5-like protein sequences are also found in specific classes of animals but not in any plants, protists, archaea, or eubacteria we could identify (Fig. 3A). As with fungi and oomycetes, the animal proteins are largely predicted to be extracellular with GPI anchors and to exhibit the signatures of SOD5-like SODs defined above. However, in animals the SOD5-like protein sequences are not 20–30-kDa SOD enzymes but rather protein domains in much larger polypeptides of ≈ 100 kDa we define as CSRP (copper-only SOD repeat proteins). An example is illustrated in Fig. 3C with CSRP of the zebrafish *Danio rerio* (XP_001343650.5). The protein is predicted to contain four tandem repeats of SOD5-like SOD domains separated by very short linkers. Each domain contains the Greek key β -barrel fold structure, disulfide cysteines, active-site arginine, and equivalents to SOD5 Glu/Gln-110 and Asp-113, except for domain four, which has a methionine instead of the Glu-110 equivalent. All but the fourth domain retain the four copper-binding histidines (Fig. 3C, top and middle). The ESL is missing, as is the zinc site in all four domains of the zebrafish CSRP. Modeling of zebrafish CSRP shows how similar each domain is to the *C. albicans* SOD5 prototype, including the positioning of the predicted copper site in domains 1–3 (Fig. 3B and 3C, bottom). It is curious that the fourth domain is very similar to SOD5 in overall fold and positioning of the disulfide and active-site arginine but has no copper site (Fig. 3C, middle and bottom). This identical pattern of

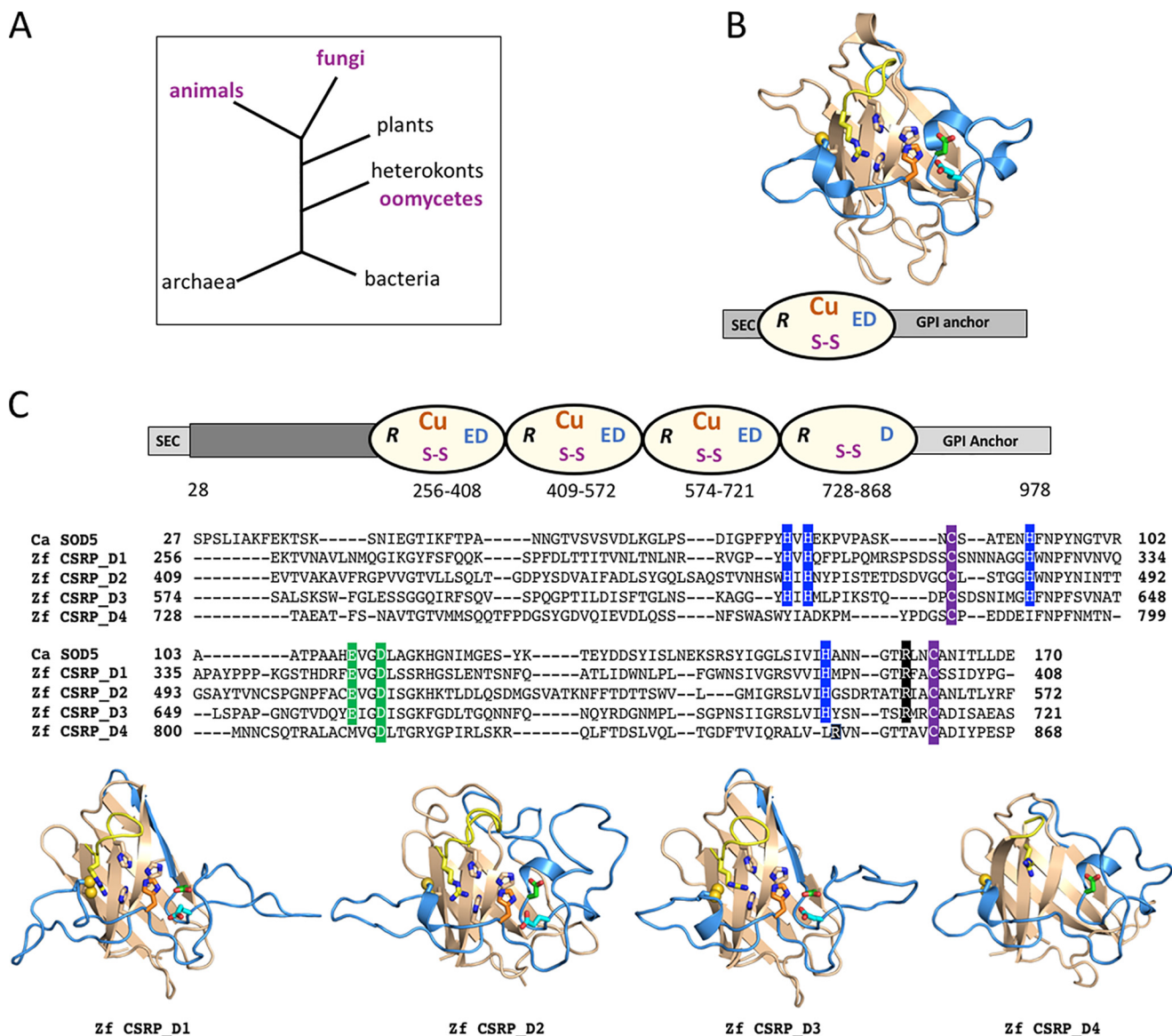


Figure 3. Evolution of copper-only SOD domains. *A*, phylogenetic tree of the distribution of copper-only SOD5-like domains in animals, fungi, and oomycetes (purple). *B*, three-dimensional structure of *C. albicans* apo-SOD5 is shown above the schematic of the full-length native protein where the N- and C-terminal sequences for secretion and GPI anchorage are in light gray and the Greek key β -barrel domain is depicted as an oval with active-site arginine (R), copper site, disulfide cysteines (S-S), and active-site Glu-110 and Asp-113 (ED). *C*, top, schematic of the predicted full-length *D. rerio* zebrafish (Zf) CSRP (XP_001343650.5) where the individual SOD5-like domains and key features are highlighted using the same scheme as for *C. albicans* SOD5 in *B*, bottom. *D. rerio* CSRP contains additional sequences at the N terminus (dark gray) of unknown nature. The ω -site for the GPI anchor is predicted to be at residue 955. *C*, middle, alignment of the individual SOD5-like domains of *D. rerio* CSRP against *C. albicans* SOD5. The copper-binding histidines are in blue; disulfide is in purple; active-site arginine is in black; and positions equivalent to SOD5 Glu-110 and Asp-113 are in green. The overall amino acid identity and similarity compared with SOD5 is as follows: CSRP_D1, 33% identity and 51% similarity; CSRP_D2, 30 and 47%; CSRP_D3, 31 and 56%; and CSRP_D4, 27 and 43%. *C*, bottom, each of the four SOD5-like domains were modeled onto *C. albicans* SOD5 using the MPI bioinformatics toolkit (93, 94). The most C-terminal repeat (CSRP_D4) is lacking a copper site but retains the predicted overall fold as well as other hallmark features of copper-only SOD5-like domains, as indicated.

three copper-binding repeats followed by a fourth non-copper-binding domain appears preserved within the class of bony fishes/Osteichthyes, e.g. CSRPs from red piranha (XP_017575212.1), common carp (KTF72519.1), and Atlantic salmon (XP_014036762.1).

CSRPs occur throughout the animal kingdom from the unicellular *Capsaspora owczarzaki* (KJE90024.1) to diverse marine invertebrates and insecta to vertebrate teleosts. In fact, at least one study looking at the evolution of SODs remarks on the presence of repeated SOD domains in *Anopheles gambiae* (82). These CSRPs have been annotated widely as Cu,Zn-SODs, but

they are clearly more related to fungal copper-only SODs. Expression of the transcripts have been analyzed, e.g. the Pacific oyster *Crassostrea gigas* CSRP (83) (EKC41617.1), and the proteins are produced as has been shown in proteomic analysis of placozoans (XP_002114624.1, Uniprot. No. B3S3A9) (84). Interestingly, CSRPs are not uniformly distributed in animals, and to date all CSRPs we have identified are in aquatic organisms and winged insects. We have yet to identify CSRP in lunged animals, e.g. avians, reptiles, and mammals. The significance of this distribution is currently not understood as the function of these curious SOD-like repeat proteins remains a

mystery. Do these multidomain CSRPs function similarly to their smaller, single-domain fungal counterparts in disproportionating superoxide, or have they evolved with an entirely distinct activity in animals? The possible function of animal CSRPs in the metabolism or sensing of reactive oxygen species and/or metals is worthy of investigation.

Concluding remarks

The eukaryotic copper-only SOD protein is not just a single unit SOD enzyme, but a protein domain conserved in evolution since the split of animals and fungi ≈ 1.5 billion years ago (Fig. 3A). In virtually all cases examined so far, including fungi, oomycetes, and animals, the copper-only SOD-like protein is predicted to be outside the cell, therefore serving in some capacity involving the environment. The fungal copper-only SOD enzyme is as fast as its Cu,Zn-SOD sister and can protect fungal pathogens from host oxidative insults as well as operate in signaling processes involving fungus-derived superoxide. The function of animal CSRPs is currently unknown, but there is precedence for diversification of small copper-binding proteins. For example, copper-binding ATX1/ATOX functions as either a single-domain ≈ 8 -kDa copper chaperone (85, 86) or as one of three domains in the copper chaperone CCS (87, 88) or as repeated protein domains in copper-transporting ATPases (89–92). Similarly, the SOD5-like copper-binding domain may have been diversified in evolution to function in numerous capacities for metal and redox homeostasis.

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THEMATIC MINIREVIEW: Copper-only SOD enzymes and protein domains

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