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Chemical warfare at the microorganismal level: a closer look at the SOD enzymes of pathogens

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Abstract

Superoxide anion radical is generated as a natural byproduct of aerobic metabolism, but is also produced as part of the oxidative burst of the innate immune response design to kill pathogens. In living systems, superoxide is largely managed through superoxide dismutases (SODs), families of metalloenzymes that use Fe, Mn, Ni or Cu cofactors to catalyze the disproportionation of superoxide to oxygen and hydrogen peroxide. Given the bursts of superoxide faced by microbial pathogens, it comes as no surprise that SOD enzymes play important roles in microbial survival and virulence. Interestingly, microbial SOD enzymes not only detoxify host superoxide, but may also participate in signaling pathways that involve reactive oxygen species derived from the microbe itself, particularly in the case of eukaryotic pathogens. In this review, we will discuss the chemistry of superoxide radicals and the role of diverse SOD metalloenzymes in bacterial, fungal, and protozoan pathogens. We will highlight the unique features of microbial SOD enzymes that have evolved to accommodate the harsh lifestyle at the host-pathogen interface. Lastly, we will discuss key non-SOD superoxide scavengers that specific pathogens employ for defense against host superoxide.

Keywords

superoxide dismutase; host-pathogen interface; redox biology; hydrogen peroxide; NADPH oxidase

The origins of superoxide in biology and in infectious disease

Two to three billion years ago, cyanobacteria evolved a remarkable capacity to split H_2O, and the O_2 gas emitted from this reaction dramatically altered the chemical composition of the planet and the course of evolution [1]. With O_2 in the atmosphere, organisms evolved methods to harness energy through O_2 reduction, providing the fuel to drive evolution of multicellularity [2–3]. Yet in biological systems, O_2 can also be detrimental through its conversion to reactive oxygen species (ROS), including superoxide ($O_2^{\bullet-}$), hydrogen

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peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$). $O_2^{\cdot-}$ is generated from the single electron reduction of O_2 and while $O_2^{\cdot-}$ cannot generally cross biological membranes, it becomes membrane permeable if protonated to hydroperoxyl ($HO_2^{\cdot}$) and can dismutate spontaneously to generate H_2O_2 and O_2. H_2O_2 can also cross biological membranes and may be further reduced through metal-catalyzed Fenton chemistry to generate the highly reactive $^{\cdot}OH$. These ROS have the capacity to oxidize virtually all organic as well as many inorganic components of the cell, and the effects can range from mild reversible modifications to protein thiols, to irreversible and lethal damage to DNA, enzymes, and cellular membranes. There are many excellent reviews on the topic of ROS in biology [4–10]. Here we shall focus on the first product of oxygen reduction, $O_2^{\cdot-}$, its sources and reactivity in biology, and the special circumstances surrounding $O_2^{\cdot-}$ at the host-pathogen interface.

All aerobic organisms generate $O_2^{\cdot-}$ as a natural byproduct of metabolism, and leakage of electrons from the respiratory chain is a just one origin of intracellular $O_2^{\cdot-}$ [11]. Aside from these endogenous or metabolic sources, microbial pathogens are faced with exogenous insults of $O_2^{\cdot-}$ from the host. As part of the innate immune process, host phagocytic cells produce $O_2^{\cdot-}$ deliberately as a means of defense against invading microorganisms and the pathogen must efficiently remove this $O_2^{\cdot-}$ before formation of species that are even more reactive. The so-called oxidative burst generated by macrophages and neutrophils involves activation of transmembrane NADPH oxidase (NOX) enzymes that use electrons from NADPH to reduce oxygen to $O_2^{\cdot-}$ [12]. The $O_2^{\cdot-}$ is produced vectorially away from the host cell cytosol in the direction of the invading pathogen either into the extracellular space or the phagolyosomal compartment, an intracellular vesicle resulting from the engulfment of microbes by phagocytes [12–13]. There are four NOX isoforms in humans. Each is a multi-subunit complex that is membrane bound and utilizes NADPH, $FADH_2$, and two molecules of heme to shuttle electrons to O_2. Catalysis is accomplished by flavocytochrome b_{558}, which is comprised of a 91 kDa glycoprotein (gp91) and a non-glycoslyated 22 kDa subunit (p22). $gp91^{phox-/-}$ mice missing the gp91 subunit of phagocytic NOX (phox) can no longer produce bursts of $O_2^{\cdot-}$ and have a greater susceptibility towards infection by bacteria and fungi [14–15].

$O_2^{\cdot-}$ on its own can be toxic to pathogens. For example, $O_2^{\cdot-}$ can disrupt microbial Fe-S clusters, elevate reactive iron levels, cause mismetallation of mononuclear Fe enzymes, or it can be converted to other reactive species to attack the invading microbe [16–20]. Phagocytic cells can also produce nitric oxide ($NO^{\cdot}$) from nitric oxide synthase (NOS) [21], a freely diffusible and labile radical that can readily react with $O_2^{\cdot-}$ generated by neighboring NOX enzymes to produce highly reactive peroxynitrite ($ONOO^-$) [22] (Scheme 1). ONOO- in turn can oxidize and damage polypeptide side chains, DNA, and transition metal centers and, if protonated, can cross biological membranes [23–24]. Another possible fate of $O_2^{\cdot-}$ in the immune response is its reduction to the membrane permeable H_2O_2, which can either react on its own with microbial macromolecules or be converted to the highly reactive $^{\cdot}OH$ through Fenton chemistry [25–26]. Additionally, in neutrophils, H_2O_2 can be converted to the membrane permeable hypochlorous acid (HOCl) via myeloperoxidase [27]. HOCL can trigger oxidative unfolding of proteins [28] and is genotoxic to microbes[29]. Overall, through the production of these various reactive oxygen and nitrogen species, $O_2^{\cdot-}$ lies at the heart of immune cell chemical warfare (Scheme 1).

ROS management through superoxide dismutase enzymes

In virtually all living organisms, $O_2^{\cdot-}$ is managed through the action of a single enzyme class, the superoxide dismutase (SOD) metalloenzymes that catalyze the two-step disproportionation of $O_2^{\cdot-}$ anion to H_2O_2 and O_2 (Scheme 2).

Since the initial discovery of a Cu-containing SOD by Fridovich and colleagues in 1969, there has been an explosion in the literature regarding the multiple families of SOD enzymes with varying metal cofactors and their multifaceted roles in ROS biology [30–32]. Due to their widespread importance, SOD enzymes have evolved on several occasions to utilize different redox-active metal cofactors. Importantly, when these proteins are substituted with other metals they typically become inactivated.[33] This occurs because the protein must be able to specifically tune the redox potential of the active site metal to accommodate its reduction midpoint potential for catalysis[34].

The most ancient of these enzymes are thought to have utilized Fe as the metal cofactor based on the abundant bioavailability of ferrous Fe in primordial oceans [35–36]. The large Fe-containing family has since expanded to also include Mn-SODs and in some instances "cambialistic" SODs that can function with either Fe or Mn (see details below) [36–40]. However, cambialistic SODs tend to have lower enzyme activity for the reasons described above[31]. Unrelated families of Cu-containing and Ni-containing SODs have also evolved over time [31, 41–42], and this expansion of cofactor options in SOD enzymes is likely linked to geological changes in metal bioavailability that accompanied oxygenation of the biosphere [43–44]. Given the virtually ubiquitous existence of $O_2^{\cdot-}$ in aerobic organisms, it is of no surprise that SOD enzymes are found in every domain of life and have even been discovered in viral genomes [45]. Importantly, since $O_2^{\cdot-}$ is not freely diffusible across membranes, each compartment requires its own SOD enzyme. In the case of microbial pathogens, the extracellular SODs are particularly important as they must directly engage the $O_2^{\cdot-}$ produced by host NOX. Below we summarize the various ways microbial pathogens have evolved to exploit SOD enzymes for survival and pathogenesis.

Bacterial Pathogens

Bacterial pathogens have been reported with Mn-, Fe-, and Cu-containing SODs, typically referred to as SodA, SodB, and SodC respectively. With few exceptions (listed below) SodA and SodB are restricted to intracellular/cytoplasmic compartments while Cu-containing SodC is exclusively in the extracellular/periplasmic space. In bacteria, Cu is generally excluded from the cytosol and bacterial cuproenzymes, including Cu-containing SodC, are restricted to extracytoplasmic compartments [46–49].

Extracellular Bacterial SODs

The extracellular/periplasmic SODs of bacteria are particularly important in that they constitute the first line of defense against the $O_2^{\cdot-}$ attack of host cells. With gram-positive bacteria, the extracellular SODs can be in direct contact with host $O_2^{\cdot-}$. The periplasmic SODs of gram-negative bacteria can also remove host $O_2^{\cdot-}$ that crosses the bacterial outer membrane through either porins or a membrane that has been permeabilized by

antimicrobial peptides[50–51]. Additionally in the low pH of the macrophage phagolysosome, protonation of host $O_2^{\cdot-}$ will facilitate its crossing of the bacterial outer membrane[52]. The vast majority of what is known regarding extracellular SODs and bacterial pathogenesis has stemmed from studies on Cu-containing SodC. Bacterial SodC enzymes are closely related to the Cu/Zn-SODs of eukaryotes in terms of overall protein fold, an intramolecular disulfide bond, and an active site that contains both the catalytic Cu and a Zn ion that assists in Cu-catalysis and helps stabilize the protein[53]. SodC can exist in a soluble form in the periplasmic space of gram-negative bacteria [46, 54] or can be tethered to the cell surface through lipid moieties [55]. In studies conducted in gram-negative bacteria, SodC is exported from the bacterial cytosol through the "Sec" pathway for unfolded proteins and arrives in the periplasm in an apo form that is then activated in this compartment through Cu and Zn insertion and disulfide oxidation [56–57] (Figure 1). There are numerous examples where loss of SodC results in decreased pathogenesis of gram-negative bacteria [54, 58–63], firmly establishing SodC as virulence factor.

Interestingly, some bacterial pathogens express multiple forms of SodC. For example, the highly virulent *E. coli* strain O517:H7, the causative agent of hemorrhagic colitis, has three SodC encoding genes [64]. One (denoted SodC) is highly similar to the SodC of non-pathogenic *E. coli*, while the other two (SodC-F1, and SodC-F2) were acquired from lambdoid phage and have biophysical properties that render them more resistant to the harsh environment of the host. SodC-F1 and SodC-F2 proteins are dimeric compared to monomeric SodC, are more resistant to proteases and low pH, and more stably bind their Cu cofactor compared to SodC [64]. Additionally, SodC-F1 and SodC-F2 isomers contain a lipid modification that helps tether the proteins to the bacterial membrane unlike the soluble SodC[64]. These unique SodC-F1 and SodC-F2 isoforms are expressed in anaerobic log phase cultures, while SodC is expressed exclusively in aerobic cultures that are in stationary phase[64]. SodC-F1 and SodC-F2 are also favorably induced in intracellular environments of an animal host cell [64]. A similar set of SodC isoforms has been well studied in *Salmonella enterica.* This food-born pathogen expresses two Cu/Zn SODs in the periplasm (SodCI and SodCII), only one of which (SodC1) contributes to virulence *in vivo*. SodCI appears to be the equivalent to the aforementioned SodC-F isoforms and becomes tethered to the membrane by binding to peptidoglycan and is more stable against protease degradation [65–69]. SodCI is particularly important for allowing *Salmonella enterica* survival in host macrophages [70].

A particularly unusual form of SodC is expressed in *Mycobacterium tuberculosis,* the causative agent of tuberculosis. Interestingly, SodC of *M. tuberculosis* is Cu-only, as there is no Zn binding site in the enzyme and no requirement for Zn in catalysis [71]. To date, *Mycobacterium* species are the only bacteria known to express Cu-only SODs; all other SodC enzymes are documented as Cu/Zn-SODs [72]. In *M. tuberculosis,* the Cu-only isoform may offer an advantage to the pathogen under conditions of fluctuating Zn pools in the host (see below, section 4.1)

SodC is not the only form of extracellular SOD in bacteria. Certain species express Mn- or Fe-containing SODs that are secreted to help defend against the $O_2^{\cdot-}$ burst of the host. In some cases, the Mn or Fe-SOD can work together with SodC to maximize oxidative stress

defense as has been shown in *M. tuberculosis* [73–76] and related *Mycobacterium* [77–78], as well as in spores of *Bacillus anthracis*, the causative agent of anthrax [79]. In other cases, the bacterium lacks an obvious SodC and the Mn- or Fe-SOD is the sole defense against extracellular $O_2^{\cdot-}$. Examples of this strategy include the secreted Mn-SodA of the *Streptococcus pyrogenes,* a causative agent of skin and mucosal infections [80–81], and the Fe-SodB of the plant pathogen *Agrobacterium tumefaciens* [82]. Although Mn- and Fe-containing SODs are highly similar enzymes at the primary sequence and tertiary structure level, they appear to use distinct modes for protein secretion and acquisition of their metal cofactors (Figure 1). With Mn-SodA, the polypeptide is exported in an unfolded apo state through the Sec pathway and the Mn cofactor is then acquired in the extracellular/periplasmic location [75], precisely as is done with Cu containing SodC [56–57] (Figure 1). By contrast, the closely related Fe-SodB can acquire its Fe cofactor before exiting the cytosol, and the enzyme is exported through the TAT system in a mature folded state, arriving outside the cell in an enzymatically active form [82–83] (Figure 1). The rationale for distinct modes of secreting SodA and SodC versus SodB may reflect bioavailability of their corresponding metal cofactor. In bacteria, Cu is known to be largely bioavailable in extracellular locations [84], and the same may be true for Mn in those species that secrete SodA, while Fe availability for SodB is prevalent in the intracellular/cytosolic location.

Intracellular SODs and bacterial pathogenesis

Intracellular SODs in pathogens are thought to act mostly on $O_2^{\cdot-}$ generated as byproducts of aerobic metabolism. Since $O_2^{\cdot-}$ is not generally thought to cross biological membranes, the intracellular SODs are not expected to defend against the oxidative burst of the host immune system. Nevertheless, many cytosolic Fe- and Mn-containing SODs have been shown to be virulence factors for bacterial pathogens [59, 79, 85–95]. Why are intracellular SODs important for bacterial pathogenesis when they are not in direct contact with host $O_2^{\cdot-}$? Even more paradoxical, the intracellular SODs are typically not required for growth under laboratory conditions, but only in the context of the host [85, 94]. It is possible that at low pH, as may be encountered in the host macrophage phagolysosome, host derived $O_2^{\cdot-}$ can be protonated to $HO^{\cdot}_2$ which can cross biological membranes, thereby providing a substrate for intracellular SODs [52]. This is feasible given the pKa of $HO_2^{\cdot}$ is 4.88 and it is known that the macrophage phagolysosome can be acidified below that pH. However, studies with *Salmonella* have argued that phagocytic superoxide specifically reacts with extracytosolic and not intracellular targets[67]. As a more potent oxidant than superoxide anion, protonated superoxide is likely to oxidize and damage molecules in extracytoplasmic compartments, without crossing the membrane. Alternatively, the intracellular SODs may not react with host derived $O_2^{-\cdot}$. Instead, metabolic constraints experienced in a host setting may alter the endogenous intracellular $O_2^{-\cdot}$ generated by the bacteria, placing a greater demand on intracellular SODs, although this has not yet been demonstrated. Finally, it is possible that certain SODs presumed to be intracellular may actually shuttle to the cell surface or are secreted in specific instances.

Obligate anaerobic bacteria that thrive in low oxygen represent an interesting case in that the endogenous metabolic production of $O_2^{-\cdot}$ appears to be lower than that of aerobic bacteria[96]. Nevertheless these microbes can be transiently subjected to oxygenated environments and

assaulted by ROS of the innate immune response and have therefore evolved with SOD enzymes as part of an oxidative stress defense, similar to aerobic bacteria[97–98]. The oxidative stress challenges of anaerobic bacterial pathogens have been reviewed elsewhere[99–100].

Clever adaptations to handling $O_2^{\cdot-}$ in the harsh metal environment of the host

A bacterial pathogen is completely dependent on its host for acquiring the metal cofactors for its SOD metalloenzymes. This acquisition of metals can be quite challenging. During infection, the host attempts to restrict metal nutrients such as Mn, Cu and Fe from pathogens or attacks the microbes with high doses of metal toxicity. Many excellent reviews have been written on the topic of metal extremes at the host-pathogen interface [101–106]. Here we provide some examples of how bacteria and fungi (see ahead) have adapted to these extremes to maintain oxidative stress protection.

As mentioned above, bacterial SodA and SodB are closely related enzymes and most Mn/Fe-SODs can bind either metal. However, mis-incorporation of the wrong metal (ie., Fe in the active site of SodA or vice versa) usually results in an inactive enzyme due to differences in redox tuning[33–34]. Nevertheless, there exists a subset of bacterial Mn/Fe-dependent SOD enzymes that are capable of maintaining SOD activity with either metal, so called cambialistic SODs [38, 40, 107]. Cambialistic SODs have been shown to be important for the virulence of some pathogenic bacteria[38]. One example involves the cytosolic SodA and SodM of the skin and soft tissue pathogen *Staphylococcus aureus*. During host invasion, *S. aureus* is subjected to severe Mn limitation caused by the host's metal-binding protein calprotectin[108]. Although Mn-SodA is vulnerable to Mn deprivation, *S. aureus* maintains intracellular SOD activity through SodM, a cambialistic SOD that switches to utilizing Fe as its cofactor under Mn-limiting conditions [38]. Another example includes the secreted Mn-SodA of *M. tuberculosis* that appears to maintain activity with Fe as its cofactor and is thus also classified as cambialistic [73]. The use of cambialistic SODs may be widespread among bacteria faced with host-imposed metal nutrient restriction.

SOD metalloenzymes are not the only means of $O_2^{\cdot-}$ defense for pathogenic bacteria as certain species have evolved non-SOD methods for dealing with host $O_2^{\cdot-}$. *Neisseria gonorrhoeae,* the causative agent of gonorrhea, derives most of its resistance to $O_2^{\cdot-}$ stress by accumulating high levels of non-proteinaceous complexes of Mn that can serve as anti-oxidants [109]. Many articles have been written on the topic of Mn antioxidants as alternatives to SODs in oxidative stress protection [110–112]. Other bacteria have found a way to take advantage of host-imposed Cu toxicity by forming non-SOD antioxidants. As mentioned above, the host can either withhold metals from microbes or attack pathogens with metal toxicity, a classic example being the Cu burst of macrophages [84, 113–115]. Yersiniabactin, a siderophore so named for its discovery in *Yersinia* sp, is a virulence factor in *Yersinia pestis*, the causative agent of the black plague[116–117]. It is also secreted from uropathogenic *E. coli* where it has been shown to bind host copper, and in doing so, generate a SOD-mimic that can protect the bacteria against host-derived $O_2^{\cdot-}$ [118–119]. Yersiniabactin may be used similarly in other species including *Y. pestis.* In spite of the extremes in metal bioavailability

at the host-pathogen interface, successful pathogens have evolved methods of adapting and even exploiting fluctuations in metals to optimize oxidative stress defenses.

Fungal Pathogens

Fungal pathogens are eukaryotic and thus require SOD enzymes within numerous intracellular organelles to accommodate the many origins of $O_2^{\cdot-}$. Unlike bacteria, where Cu-containing SODs are exclusively extracellular, the major Cu-containing SOD of fungi and other eukaryotes is intracellular. This Cu/Zn-SOD, typically known as SOD1, is largely cytosolic and also present in the intermembrane space of the mitochondria, mimicking the periplasmic location of bacterial Cu-containing SODs[46, 120–121]. Within the matrix of the mitochondria is a separate Mn-containing SOD2, and the combined action of SOD1 and SOD2 effectively remove $O_2^{\cdot-}$ produced during mitochondrial respiration. Although eukaryotic Mn-SODs are typically mitochondrial, there are rare cases of cytosolic Mn-SODs in fungal pathogens as described below. In addition to the aforementioned intracellular SODs, many fungal pathogens also express extracellular SODs. Analogous to the extracellular SODs of bacteria pathogens, these fungal SODs represent the first line of defense against host-derived $O_2^{\cdot-}$.

Extracellular Fungal SODs

Fungi produce an unusual form of extracellular SOD that is Cu-only and does not require Zn, similar to that described for *Mycobacteria* above [72]. These SODs can be either attached to the cell wall through GPI-anchors or secreted[122–123]. Interestingly, Cu-only SODs are the only type of extracellular SOD in the fungal kingdom and there have been no reports of Cu/Zn-, Mn-, or Fe-containing SODs secreted from any fungal species[72]. Cu-only extracellular SODs are widespread among fungal pathogens and in fungal-like oomycetes species [72, 124–125]. Like *Mycobacterium* Cu-only SODs, the fungal SODs are missing two histidine residues necessary to coordinate zinc in the active site, and additionally lack protein sequences spanning a structure known as the electrostatic loop (ESL), which in Cu/Zn SODs helps channel the $O_2^{\cdot-}$ substrate and stabilize metal binding to the enzyme[126124–125]. Because they lack an ESL covering the active site, the Cu site of fungal Cu-only SODs is uniquely solvent exposed. Despite these deviations from Cu/Zn-SODs, fungal Cu-only SODs are highly active enzymes with rates of $O_2^{\cdot-}$ disproportionation that reach diffusion limits ($1.8 \times 10^9 M^{-1}s^{-1}$) [124–125].

Another distinguishing feature of fungal Cu-only SODs is their mode of enzyme activation through Cu-insertion. In animals, the extracellular SOD is a Cu/Zn-SOD that acquires its metal cofactors in the secretory pathway and arrives at the cell surface in a fully active state [127–128]. By comparison, the fungal Cu-only SODs can be loaded with Cu outside of the cell [125], similar to what is observed in bacteria for the periplasmic Cu/Zn SOD [56] and (Figure 1). During infection, the host can attack pathogens with high concentrations of Cu and the microbe may, in turn, use this host Cu to charge its extracellular SODs to defend against $O_2^{\cdot-}$ produced by the host [113–115, 129]. Since Cu-only SODs have no requirement for Zn, their activity is not impacted by Zn bioavailability, which can vary greatly within the host during infection [102, 130–132].

Extracellular Cu-only SODs have been shown to be virulence factors for pathogenic fungi. The best studied are those in the opportunistic fungal pathogen, *Candida albicans. C. albicans* encodes three extracellular Cu-only SOD enzymes (SOD4, SOD5, and SOD6). During host invasion, SOD4 and SOD5 are both abundantly expressed, suggesting they play important roles in eliminating $O_2^{\cdot-}$ produced at sites of infection [133–136]. SOD5 has been shown to be a virulence factor for *C. albicans* in a systemic model of infection and is important for fungal survival against the oxidative attack of macrophages and neutrophils [137–139]. Why *C. albicans* encodes three different extracellular Cu-SOD enzymes is currently not understood, but each SOD may function in distinct host niches. In addition to *C. albicans,* the pulmonary fungal pathogen *Histoplasma capsulatum* expresses a single Cu-only SOD3 that has been shown to be important for fungal survival against the $O_2^{\cdot-}$ attack of macrophages and for virulence in a mouse model of lung infection[122, 140]. Cu-only SODs are also important for virulence of the pulmonary fungal pathogen *Paracocidiodes brasiliensis*[141] and of the arthropod fungal pathogen *Beauveria bassiana*[142] that can cause keratitis in humans [143–144]. It is important to note that not all fungal pathogens have extracellular SOD enzymes, an example being *Cryptococcus neoformans,* a pulmonary and central nervous system pathogen. Interestingly, the largely cytosolic Cu/Zn-SOD1 of *C. neoformans* can be enriched in lipid rafts and may shuttle to the cell surface to deal with host-derived ROS [145].

$O2^{\cdot-}$ produced by host cells may not be the only substrate for fungal extracellular SODs. Recent studies have shown that *C. albicans* produces a burst of extracellular $O_2^{\cdot-}$ by a fungal NOX enzyme (FRE8) during morphogenesis and that this $O_2^{\cdot-}$ serves as a substrate for extracellular Cu-only SOD5. The diffusible H_2O_2 product generated by SOD5 then acts as a signal to modulate morphogenesis through a mechanism that is currently not known [146]. In fact, SOD5 is specifically induced during the fungal oxidative burst, indicating that SOD5 may have evolved to deal with fungal-derived ROS [138]. Overall, the role of Cu-only SODs at the host-pathogen interface is complex: these SODs can both protect the fungus from host-derived $O_2^{\cdot-}$ as well as function in fungal signaling pathways involving ROS (Figure 2). In any case, these SODs are important for fungal virulence and based on the biophysical properties that distinguish them from the Cu/Zn SOD of humans, this unique class of SODs represent a possible therapeutic target for treating fungal infections.

Intracellular Fungal SODs

Intracellular SODs of fungi have also been shown to be virulence factors. Deletion of the mitochondrial matrix SOD2 reduces virulence in mouse and insect models of *C. neoformans* and *B. bassiana* infection, respectively, likely due to the importance of detoxifying ROS byproducts of respiration [147–149]. Cu/Zn-SOD1 has been shown to be a virulence factor in mouse models for both *C. albicans* and *C. neoformans* infection and is also important for fungal survival in macrophages [150–152]. As described above for bacterial pathogens, the requirement for intracellular SODs during infection may reflect the protonation and subsequent membrane permeability of host-derived $O_2^{\cdot-}$, or changes in fungal production of metabolic $O_2^{\cdot-}$ to accommodate life within the host. It is also possible that the intracellular SODs shuttle to the cell surface to remove host derived $O_2^{\cdot-}$, as proposed above for *C. neoformans* SOD1[145]. Lastly, as described in more detail below with protozoans,

intracellular SODs may participate in cell signaling pathways that involve ROS and drive pathogenesis.

Since SOD enzymes are metalloproteins, microbes must adapt to changes in host metals to maintain SOD activity. As described above, the *S. aureus* bacterium adapts to low Mn availability by substituting Fe as a cofactor for Mn-SOD enzymes[38] and a similar process of metal cofactor swapping has been described for the cytosolic SODs of certain fungal pathogens. Specifically, *C. albicans* and closely related fungi express a rare form of Mn-SOD (SOD3) that resides in the cytosol, the same location as Cu/Zn-SOD1. *C. albicans* will switch from expressing Cu/Zn-SOD1 to cytosolic Mn-SOD3 when Cu is low, a condition that occurs in the kidney during fungal infection [153–154]. This adaptation allows *C. albicans* to maintain cytosolic SOD activity even in times of Cu depletion[153–154]. The arthropod fungal pathogen *B. bassiana* also contains dual cytosolic Mn and Cu/Zn-containing SODs and, moreover, expresses a pair of mitochondrial matrix SODs that use either Mn or Fe as a cofactor. Mitochondrial Fe-SODs are extremely rare among fungi and *B. bassiana* may alternate between Fe and Mn SODs to accommodate changes in availability of these metals. All five of the *B. bassiana* SODs are important for virulence, including the extracellular Cu-only and the four intracellular Cu/Zn-, Mn- and Fe-SODs[142, 149, 155]. This large repertoire of SOD enzymes with varying metal cofactors may aide in the pathogens ability to infect many different niches in diverse arthropods as well as humans.

Protist Pathogens

Protozoans are unicellular eukaryotic microbes and, as is the case with pathogenic bacteria and fungi, parasitic species have evolved intriguing strategies involving SOD enzymes to promote virulence within the host. Curiously, all the SOD enzymes characterized to date in protist pathogens utilize Fe as their cofactor; there has been no documentation of Mn- or Cu-containing SODs in infectious protozoans [36]. There is some evidence for secretion of the Fe-SODs, as was shown with *Trypanosoma cruzi*, the pathogen responsible for Chagas disease [156]. Yet much of what is known on protozoan SODs focuses on the intracellular enzymes, including Fe-SODs of the mitochondria and extra-mitochondrial compartments, eg., cytosol and glycosomes/peroxisomes. These intracellular SODs have been shown to be virulence factors in multiple species[157–158].

Studies on the mitochondrial Fe-containing SOD of *Leishmania amazonensis*, a causative agent of leishmaniasis, have provided insight into how a mitochondrial SOD can be important for virulence. *L. amazonensis* switches between non-virulent (promastigotes) and virulent (amastigotes) states and morphogenesis into the virulent state is triggered by a signaling pathway involving mitochondrial ROS and mitochondrial Fe-SODA, although the mechanism is not known [157]. Removal of just one allele of SODA was sufficient to disrupt this signaling pathway, inhibit protist replication in macrophages and decrease virulence in a mouse model of cutaneous infection [157]. These findings underscore the notion that microbial SODs are not just for guarding against the oxidative attack of the host; the SODs also promote pathogenesis through signaling pathways involving ROS.

Trypanosoma cruzi has four predicted isoforms of Fe-SOD, two of which, SODA and SODB, reside in the mitochondria and cytosol respectively. Remarkably, SODB has been shown to be resistant to inactivation by ONOO-. As mentioned above (Scheme 1), macrophage attack of microbes involves formation of diffusible NO that can react with either host- or pathogen-derived $O_2^{\cdot-}$ to generate ONOO-. Fe and Mn SODs from bacteria to humans are notoriously susceptible to inactivation by peroxynitrite-mediated nitration of an active site tyrosine residue [159–160] (Figure 3). The unusual resistance of *T. cruzi* SODB to ONOO- reflects repair of nitrated Tyr35 at the active site via an intramolecular electron transfer event involving nearby Cys83 [161] (Figure 3). The closely related *Trypanosoma brucei*, the causative agent of African sleeping sickness, has also been shown to encode four highly similar Fe-SOD enzymes that may play analogous roles as the isoforms of *T. cruzi* [162].

Given the importance of the Fe-containing SODs of protozoans during infection, they have become targets of interest for drug design. Although the Fe-SODs are closely related to the Mn-SOD2 of human mitochondria, there are key differences that may be targeted. For example, in the substrate channel leading to the active site, a lysine residue caps the active site of protozoan Fe-SODs, but not that of mammalian SOD2 [163–164]. Strategies exploiting such differences between protist and mammalian SODs are being used in the design of inhibitors against *Plasmodium, Leishmania,* and *Trypanosoma sp*.[163–165], and one suspected inhibitor of *T. cruzi* cystosolic Fe-SODB was able to deplete parasite levels in a mouse infection model [166–167]. Curiously, some parasitic protozoans lack SODs altogether. Such is the case for the intestinal pathogen and anaerobe *Giardia intestinalis*, which instead contains a cytosolic superoxide reductase (SOR) [168]. SORs catalyze the conversion of $O_2^{\cdot-}$ into H_2O_2 in the presence of a reductant. They have been found in many archaea and bacteria, but *G. intestinalis* has the first SOR characterized in a eukaryote [31]. Given that SORs only cause the reduction of $O_2^{\cdot-}$ to H_2O_2 without production of O_2 like SODs, this might be a protective adaptation for an oxygen-sensitive organism such as *G. intestinalis* [169].

Concluding Remarks

It is clear that SODs are important for pathogens to protect against the oxidative burst generated by the host. These metalloenzymes have evolved multiple times to utilize different metal cofactors and pathogens have selected for SOD enzymes that maintain activity in the face of many different environments that vary widely in metal availability. In certain bacterial and fungal microbes, success of the pathogen relies on its ability to switch cofactor usage in SOD enzymes when the host attempts to starve the microbe of metal nutrients such as Mn, Fe and Cu. In most pathogens, extracellular SODs are the first line of defense against host-derived $O_2^{\cdot-}$, yet intracellular SODs are also virulence factors for bacterial, fungal and protozoan pathogens. These SODs may guard against protonated host $O_2^{\cdot-}$ that can cross the cell membrane, or may promote microbial survival by managing endogenous or metabolic ROS. Although microbial SODs clearly thwart the oxidative attack of the host, they can also promote pathogenesis in ways that do not involve oxidative stress protection. In eukaryotic pathogens, including fungi and protists, SODs can participate in signaling pathways that enhance virulence. With the many ways that microbial SODs support virulence, they have become attractive targets for drug development. Strategies currently being explored to inhibit

the Fe-SODs of protozoa may be expanded in the future to include other specialized SODs of microbial pathogens, including the Cu-only SODs of fungi that are clearly distinct from their mammalian Cu/Zn-counterpart. Lastly, it is important to remember that SOD enzymes are not the only method for handling $O_2^{\cdot-}$, and non-SOD forms of $O_2^{\cdot-}$ removal can be seen in microbial pathogens such as SORs in certain protists and non- proteinaceous SOD-mimics in bacterial pathogens. It is possible that additional mechanisms for dealing with $O_2^{\cdot-}$ during infection are yet to be discovered in successful pathogens that lack an obvious extracellular SOD enzyme.

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Abbreviations

ESL	electrostatic loop
GPI	glycosylphosphatidylinositol
NOX	NADPH oxidase
ROS	reactive oxygen species
Sec	secretory translocon
SOD	superoxide dismutase
SOR	superoxide reductase
TAT	twin-arginine translocation

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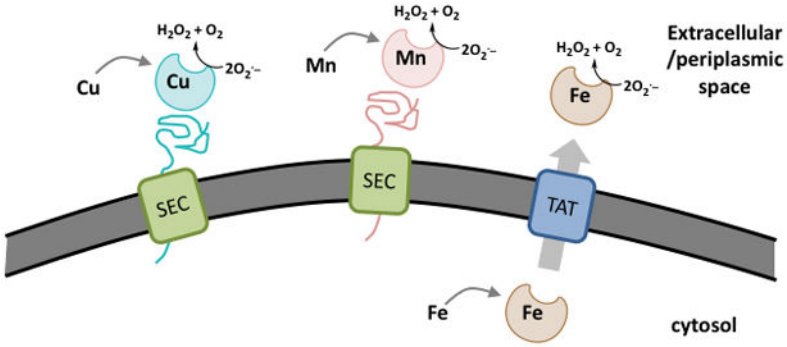


Figure 1. Bacterial secreted SODs differ in their modes of export and metal acquisition

Model showing differential secretion and metal insertion of three classes of bacterial extracytoplasmic SODs. Cu-SODs (blue) and Mn-SODs (pink) are secreted in the unfolded state by Sec translocation and become folded and activated with their respective metal cofactors in the periplasm or extracellular space. Fe-SODs (brown) appear to acquire their metal cofactor in the intracellular/cytosolic space and are translocated in the native and enzymatically active from by the TAT system.

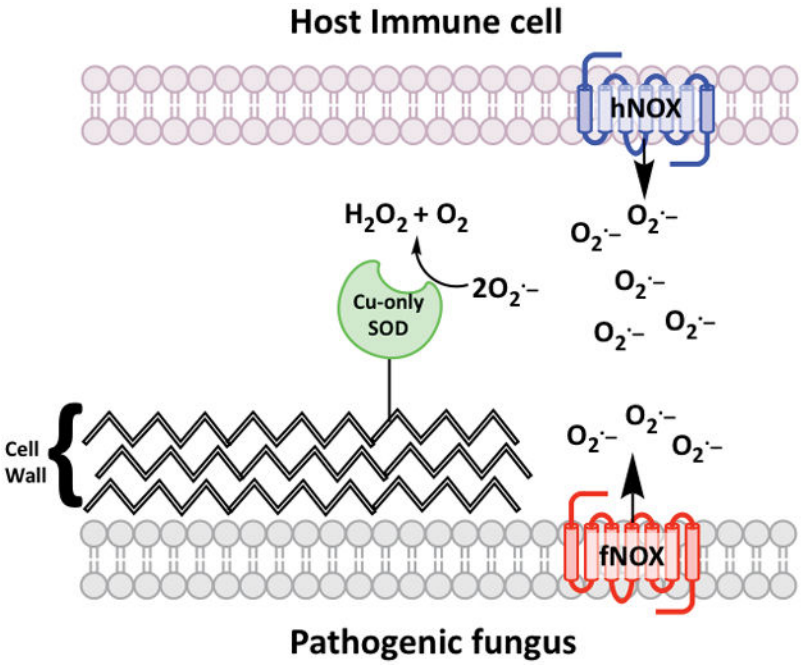


Figure 2. Fungal Cu-only SODs can react with $O_2^{\cdot-}$ from both the host and the fungus

The extracellular Cu-only SODs of fungi (green) are linked to the fungal cell wall through GPI anchors or are secreted. These SODs are known to remove $O_2^{\cdot-}$ from the host NOX enzyme (hNOX, blue) of immune cells including macrophages and neutrophils. In addition, studies with *C. albicans* have shown that Cu-only SODs can remove $O_2^{\cdot-}$ from fungal NOX enzymes (fNOX, red) and the concomitant production of H_2O_2 can promote morphogenesis of the fungus.

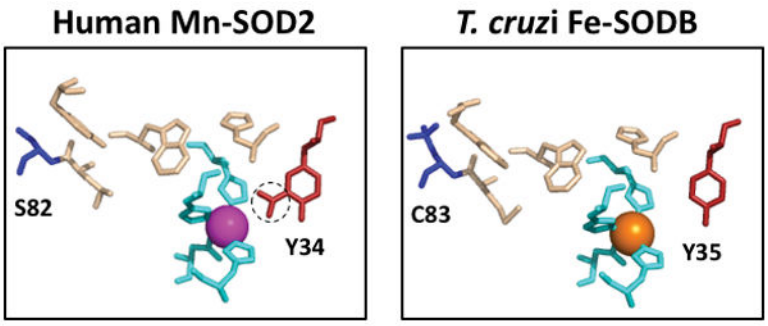
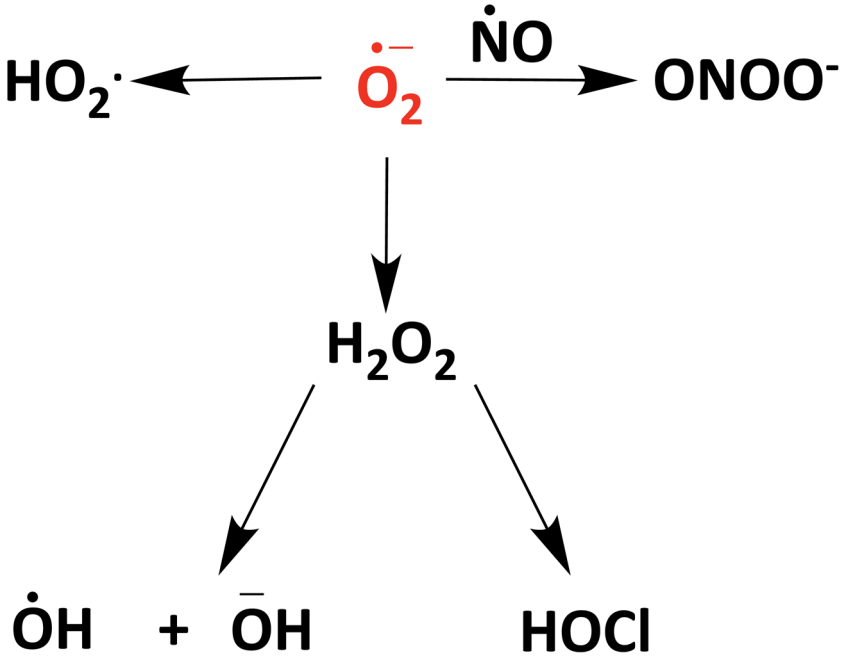
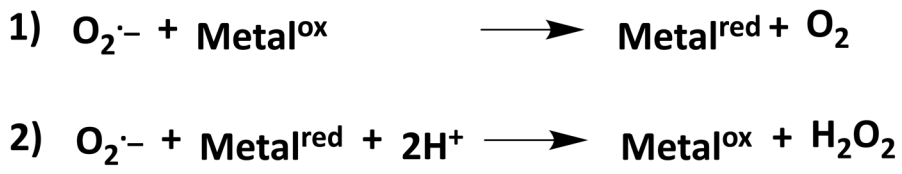


Figure 3. Resistance of *T. cruzi* Fe-SODB to inactivation by peroxynitrite

A comparison of the active site of human mitochondrial Mn-SOD2 (left) versus *T. cruzi* cytosolic Fe-SOD (right) generated using PDB ID: 2ADP and 2GPC, respectively. The Mn and Fe atoms are shown as magenta and orange balls respectively, and the metal coordinating residues are in aqua. A tyrosine near the active site (red) is nitrated in the case of Mn-SOD2 (circle), but not with *T. cruzi* Fe-SOD where the nitrated tyrosine is repaired through an intramolecular electron transfer involving cysteine 83 (blue). This cysteine is replaced by serine 82 (blue) in human Mn-SOD2.



Scheme 1.
Superoxide is central to the production of highly reactive oxygen and nitrogen species



Scheme 2.
Two half reactions for the disproportionation of $O_2^{\cdot-}$ by SOD enzymes