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Original Contribution

The superoxide reductase from the early diverging eukaryote *Giardia intestinalis*

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ABSTRACT

Unlike superoxide dismutases (SODs), superoxide reductases (SORs) eliminate superoxide anion ($O_2^{\bullet-}$) not through its dismutation, but via reduction to hydrogen peroxide (H_2O_2) in the presence of an electron donor. The microaerobic protist *Giardia intestinalis,* responsible for a common intestinal disease in humans, though lacking SOD and other canonical reactive oxygen species-detoxifying systems, is among the very few eukaryotes encoding a SOR yet identified. In this study, the recombinant SOR from *Giardia* (SOR_{Gi}) was purified and characterized by pulse radiolysis and stopped-flow spectrophotometry. The protein, isolated in the reduced state, after oxidation by superoxide or hexachloroiridate(IV), yields a resting species (T_{final}) with Fe^{3+} ligated to glutamate or hydroxide depending on pH (apparent $pK_a = 8.7$). Although showing negligible SOD activity, reduced SOR_{Gi} reacts with $O_2^{\bullet-}$ with a pH-independent second-order rate constant $k_1 = 1.0 \times 10^9\ M^{-1}\ s^{-1}$ and yields the ferric-(hydro)peroxo intermediate T_1; this in turn rapidly decays to the T_{final} state with pH-dependent rates, without populating other detectable intermediates. Immunoblotting assays show that SOR_{Gi} is expressed in the disease-causing trophozoite of *Giardia.* We propose that the superoxide-scavenging activity of SOR in *Giardia* may promote the survival of this air-sensitive parasite in the fairly aerobic proximal human small intestine during infection.

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The adventitious partial reduction of molecular O_2 in a living cell gives rise to formation of a plethora of cytotoxic species, collectively termed "reactive oxygen species" (ROS), which can impair a wide spectrum of biomolecules with key functions. To counteract oxidative damage, all living beings have thus developed enzymatic systems to accomplish ROS detoxification with high selectivity and efficacy.

Superoxide anion ($O_2^{\bullet-}$), one of the best studied ROS, is a highly reactive radical species produced by the one-electron reduction of O_2. In many (micro)organisms, this radical is detoxified to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), a well-known metalloprotein that efficiently catalyzes the following reactions:

$$O_2^{\bullet-} \rightarrow O_2 + 1e^-, \quad (1)$$

$$O_2^{\bullet-} + 2H^+ + 1e^- \rightarrow H_2O_2, \quad (2)$$

$$2O_2^{\bullet-} + 2H^+ \rightarrow O_2 + H_2O_2. \quad (1)+(2)$$

The peroxide produced is then typically eliminated by catalase:

$$2H_2O_2 \rightarrow O_2 + 2H_2O. \quad (3)$$

SOD and catalase promote the removal of $O_2^{\bullet-}$ and H_2O_2, respectively, through their dismutation, which leads to formation of molecular oxygen as a co-product. As O_2 production is clearly disadvantageous for air-sensitive microorganisms, it is not surprising that during the past decade many microaerobes or anaerobes have been shown to code for $O_2^{\bullet-}$- and H_2O_2-detoxifying enzymes that operate without releasing O_2. This is the case for superoxide reductase (SOR; see [1–5] for reviews), a nonheme iron-containing enzyme that was shown to eliminate superoxide not through its dismutation (Reaction (1)+(2)), but via reduction to H_2O_2 (Reaction (2)) [6,7] in the presence of a suitable electron donor.

Abbreviations: ROS, reactive oxygen species; SOR, superoxide reductase; SOD, superoxide dismutase; SOR_{Gi}, superoxide reductase from *Giardia intestinalis*; 1Fe–SOR, one-iron-containing SOR; 2Fe–SOR, two-iron-containing SOR; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris (hydroxymethyl)aminomethane; SVD, singular value decomposition; EDTA, ethylenediaminetetraacetate.

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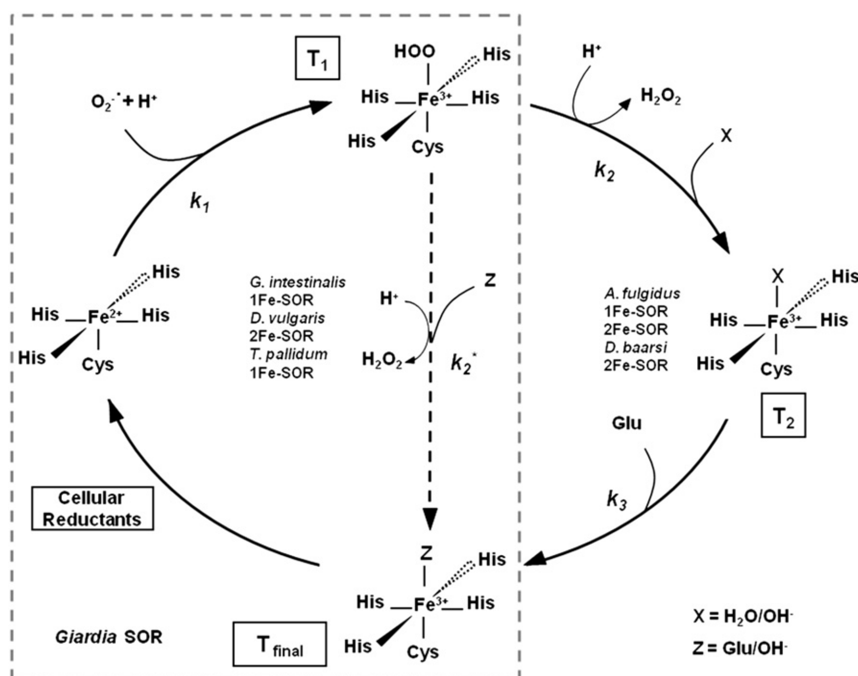
So far, two classes of SORs have been identified: 1Fe–SORs, also called neelaredoxins (from "neela," the Sanskrit word for blue [8]) because of their characteristic blue color in the oxidized form, and 2Fe–SORs (or desulfoferrodoxins [9]), which have an extra desulforedoxin-like domain fused to the N-terminus with an additional $[FeCys_4]$ center (called "center I") whose function is still elusive. The reaction of superoxide with 1Fe– and 2Fe–SORs occurs at their common iron center (called "center II" in 2Fe–SORs), which, in the reduced form of the enzyme, consists of a pentacoordinated iron(II) with four equatorial histidines and one axial cysteine in a square pyramidal geometry. In the oxidized state, a monodentate carboxylate from a glutamate residue (E14 in the enzyme from *Pyrococcus furiosus*) typically binds the $Cys(His)_4–Fe^{3+}$ center in the sixth axial position, resulting in a hexacoordinated octahedral geometry. The three-dimensional crystallographic structure of a few SORs has been determined [10–14], showing that the active iron site, located in a seven-stranded β-barrel domain, is close to the molecular surface and exposed to the solvent. Site-directed mutagenesis studies revealed that the reaction with superoxide is facilitated electrostatically by the positive charge of a solvent-exposed lysine residue (K15 in the *P. furiosus* enzyme). The aforementioned glutamate and lysine residues both lie in an EKHVP motif typically (but not strictly) conserved in SORs.

The physiological redox partner of SOR is still a matter of debate. Rubredoxins, small proteins (molecular mass of a few thousand kilodaltons) with a $[FeCys_4]$ center known to be involved in electron transfer processes, have been generally assumed to be the direct electron donors to SOR, based on the fact that the genes encoding rubredoxin and SOR lie in the same operon in some bacteria. Consistently, reduced rubredoxins were shown to reduce both 1Fe– and 2Fe–SORs with second-order rate constants on the order of 10^6–10^7 M^{-1} s^{-1} [15–17]. However, physiological electron donors other than rubredoxins must exist because rubredoxins are missing in a large number of organisms that encode SORs. Moreover, a certain degree of promiscuity in the reaction of SOR with electron donor proteins is seemingly allowed. The 2Fe–SOR gene from *Desulfoarculus baarsi* was indeed shown to complement deletion of the *sodAB* genes in *Escherichia coli* [18], in which a rubredoxin is missing. Incidentally, this observation, reported in 1996, provided the first experimental evidence for a role for SOR in superoxide detoxification. This complementation was later observed for other 2Fe– or 1Fe–SORs.

The mechanism of the reaction of superoxide with SOR has been extensively investigated mostly by pulse radiolysis [17,19–27], though recently an experimental protocol to follow the reaction by stopped-flow spectrophotometry was also reported [28]. There is general consensus that addition of superoxide to the reduced enzyme yields a first intermediate (T_1) with a maximal absorption in the 580–630 nm range, assigned to a ferric-(hydro)peroxo species (see Scheme 1). Formation of T_1 occurs at $k_1 = 10^9$ M^{-1} s^{-1} independent of pH, which indicates that if protonation of bound superoxide takes place at this step, it does not limit the reaction rate. The fate of the T_1 intermediate varies among the different SORs that have been investigated. In some enzymes (namely the 2Fe–SOR from *Desulfovibrio vulgaris* [22,23,28] and the 1Fe–SOR from *Treponema pallidum* [25]), T_1 was shown to rapidly decay, with pH-dependent rates, directly to a final species (T_{final}), with Fe^{3+} ligated to either glutamate or hydroxide, depending on pH. Glutamate/hydroxide exchange at the ferric ion was shown (i) to induce remarkable differences in the absorption spectra (up to 90-nm shift of the band in the visible region and the appearance/disappearance of a broad band at ~330 nm) and (ii) to have apparent pK_a values in the range 6.0–9.6, depending on the SOR. Conversely, in other enzymes (the 1Fe– and 2Fe–SORs from *Archaeoglobus fulgidus* [17,26] and the 2Fe–SOR from *Desulfoa. baarsi* [21]), T_1 was reported to decay to T_{final} via formation of another intermediate, called T_2, with Fe^{3+} bound to H_2O or OH^-: in these cases, the formation rates of both T_2 and T_{final} were found to be also dependent on pH.

SORs were initially thought to be restricted to the Bacteria and Archaea domains and, accordingly, SOR-encoding genes have been identified in a large number of anaerobic prokaryotes. Recently, however, the occurrence of a putative SOR-encoding gene was also suggested for the early divergent protozoan pathogen *Giardia intestinalis* [5] and a few more eukaryotes [29]. *G. intestinalis* is the amitochondriate, microaerophilic parasite responsible for giardiasis, a common intestinal infectious disease with 280 million symptomatic human



Scheme 1. Proposed mechanism for the catalytic cycle of superoxide reductases (modified from [5]). The reduced enzyme from *Giardia*, like those from *Desulfov. vulgaris* and *T. pallidum*, upon reaction with superoxide undergoes a direct transition from T_1 to T_{final} with no evidence for the formation of the T_2 intermediate.

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