Minireview

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Superoxide Anion Radical ($O_2^{\cdot-}$), Superoxide Dismutases, and Related Matters*

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A field of inquiry may be said to have come of age when conclusions initially viewed as remarkable or even unbelievable are accepted as commonplace. Study of the biology of the superoxide anion radical and of related free radicals, and the defenses thereto, has now reached this happy state of maturity. Superoxide and even hydroxyl radicals are now known to be produced in living systems, and elaborate systems of defense and repair, which minimize the ravages of these reactive species, have been described. New members of the superoxide dismutase, catalase, and peroxidase families of defensive enzymes are being found, as are new targets that are modified by $O_2^{\cdot-}$. In addition, the involvement of $O_2^{\cdot-}$ in both physiological and pathological processes is being established. A weighty tome would be needed to encompass a comprehensive coverage of this field of study. This review will describe only aspects of the biology of oxygen radicals that currently engage the interest of the writer. Hopefully they will also be of interest to the reader. Other recent reviews may serve to fill the gaps in this one (1–6).

Measurement of $O_2^{\cdot-}$ in Vitro and in Vivo

Because of the spin restriction, the univalent reduction of O_2 to $O_2^{\cdot-}$ is a facile process, and $O_2^{\cdot-}$ production by spontaneous as well as enzyme-catalyzed reactions has been demonstrated. The instability of $O_2^{\cdot-}$ in aqueous solutions is a hindrance to its detection and measurement. This has been circumvented by exploiting its reaction with various "detector" molecules such as ferricytochrome *c* or spin-trapping agents. These agents are not specific for $O_2^{\cdot-}$. Thus, reductants other than $O_2^{\cdot-}$ can reduce ferricytochrome *c*, and oxidants other than $O_2^{\cdot-}$ can convert the spin traps to their epr-detectable hydroperoxy derivatives (7, 8). Inhibition by SOD[1] is used to lend specificity to these methods.

Detection and measurement of fluxes of $O_2^{\cdot-}$ within cells is a goal as difficult as it is desirable. Unfortunately enthusiasm for achieving this goal has led many investigators to use flawed methods. An example is the luminescence that can be elicited from lucigenin. Early studies of the lucigenin luminescence elicited by the xanthine oxidase reaction led to the realization that the lucigenin dication must be univalently reduced to the corresponding monocation before reacting with $O_2^{\cdot-}$ in the process that leads to luminescence (9, 10). The chemistry involved was discussed in a more recent review (11). Nevertheless the use of lucigenin as a "specific" detector of $O_2^{\cdot-}$ continues. Quite recently it was shown that the lucigenin monocation radical can autoxidize and thus produce $O_2^{\cdot-}$, even in cases where no $O_2^{\cdot-}$ was being produced in the absence of lucigenin (12). In studies with *Escherichia coli*, lucigenin was shown to function, much as does paraquat, to increase intracellular $O_2^{\cdot-}$ production (13). Hopefully the widespread but inappropriate use of lucigenin luminescence as a measure of $O_2^{\cdot-}$ will stop.

Another luminescent method that is misused to measure $O_2^{\cdot-}$ is based on luminol. In this case the compound must be univalently oxidized to a luminol radical, which reacts with $O_2^{\cdot-}$ before the light-emitting pathway is entered upon. The problem in this case is that the luminol radical can spontaneously reduce O_2 to $O_2^{\cdot-}$. Luminol luminescence thus can be caused by a variety of oxidants and in all cases SOD inhibits (14, 15). Here again the detector is acting as a source of $O_2^{\cdot-}$.

One additional artifactual detector of $O_2^{\cdot-}$ needs to be mentioned because of its widespread misuse, and that is nitroblue tetrazolium. Many enzymes can cause the reduction of tetrazolium salts to the corresponding formazans. Reduction of nitroblue tetrazolium to the monoformazan requires two electrons and to the diformazan four electrons. When proceeding by a univalent pathway, which is usual, one encounters tetrazoinyl radical intermediates (16), which reduce O_2 to $O_2^{\cdot-}$ in a reversible process. SOD by removing $O_2^{\cdot-}$ displaces this oxidation to the right and thus prevents production of the formazan. For this reason many aerobic tetrazolium reductions are inhibitable by SOD even though $O_2^{\cdot-}$ was not being produced in the system in the absence of the tetrazolium (17).

Is there any method which can reliably be used as a measure of intracellular $O_2^{\cdot-}$? There is and it is based on the rapid inactivation of [4Fe-4S]-containing dehydratases, such as aconitase. $O_2^{\cdot-}$ oxidizes the clusters of these dehydratases resulting in loss of Fe(II), and that is reversible by reduction and reincorporating of Fe(II). The balance between these opposing processes can be used as a measure of $O_2^{\cdot-}$ and has been so used in *E. coli* (18) and in mammalian cells (19). The inactivation of aconitase by $O_2^{\cdot-}$ provides an explanation for previously inexplicable observations. Thus high pO_2 (291 mm) increased both glucose utilization and lactate production, by 4–6-fold, in WI38 cells (20). Raising pO_2 would increase $O_2^{\cdot-}$ production, and inactivation of aconitase by $O_2^{\cdot-}$ would force the cells to rely on fermentation of glucose for energy. In another example *Aspergillus niger* was reported (21) to accumulate less citrate in the medium when supplied with 0.1 mg/liter Mn(II). In this instance enrichment of the medium with Mn(II) would increase the level of Mn-SOD in the mitochondria, and that in turn would decrease $O_2^{\cdot-}$ and thus raise aconitase. The final effect would be increased metabolism of citrate via the Krebs cycle and less citrate excretion. Near UV irradiation of *E. coli* inhibited growth on succinate more than growth on glucose (22) and inhibited respiration (23). Both of these effects can be explained by the inactivation of aconitase by photosensitized production of $O_2^{\cdot-}$.

Aconitase can be inactivated by oxidants other than $O_2^{\cdot-}$. Of these peroxynitrite is particularly relevant to biology, but in NO-producing cells the level of peroxynitrite is itself dependent upon $O_2^{\cdot-}$ production. Inactivation by H_2O_2 is relatively unimportant.

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[1] The abbreviations used are: SOD, superoxide dismutase; FALS, familial amyotrophic lateral sclerosis; EC-SOD, extracellular SOD.

Oxidants from O_2^- in Vitro and in Vivo

Although O_2^- can initiate and propagate free radical oxidations of leukoflavins, tetrahydropterins, catecholamines, and related compounds and can inactivate [4Fe-4S]-containing dehydratases, it does not significantly attack polyunsaturated lipids or DNA. Yet defects in SODs, which would have the effect of raising intracellular [O_2^-], do lead to cell envelope damage (24) and to enhanced mutagenesis (25). Mechanisms by which O_2^- can give rise to more potent oxidants could explain these seeming anomalies, and there are several such mechanisms. The simplest of these is protonation to hydroperoxyl radical, whose pK_a is 4.8 and which is a much stronger oxidant than is O_2^-. Association of O_2^- with other cationic centers such as vanadate (26) or Mn(II) (27) also have this effect, but these mechanisms are unlikely to apply generally within cells.

There is a mechanism pertinent to living cells, and we may call it the *in vivo* Haber-Weiss reaction. It is a process in which O_2^- increases "free" iron by oxidizing the [4Fe-4S] center of dehydrases such as dihydroxy acid dehydrase (28), 6-phosphogluconate dehydrase (29), fumarases A and B (30), and aconitase (31, 32). The released iron is kept reduced by cellular reductants, and the Fe(II) reacts with H_2O_2, as in the Fenton reaction, to yield Fe(III) + HO$^\bullet$ or its formal equivalent, Fe(II)O. This was proposed (33) to provide an explanation for the enhanced O_2-dependent mutagenesis exhibited by *sodA sodB E. coli*, and it has been experimentally verified (34, 35). The importance of release of iron by O_2^- from [4Fe-4S] clusters of dehydrases was underscored by the recent observation of the complementation of *sodA sodB E. coli* by insertion and overexpression of rubredoxin reductase (36). Rubredoxin reductase may play a role in reconstitution of the oxidatively disassembled [4Fe-4S] clusters of dehydrases and thereby lower the "free" iron in aerobic SOD-null *E. coli*, or it may somehow scavenge O_2^- within cells.

Mutations and Complementations of Superoxide Dismutases

The primary defense against the damage that can be caused by O_2^-, and by its reactive progeny, is the SODs. The importance of these enzymes has been clarified by the phenotypic deficits of mutants defective in their production and in a number of cases by the complementing effects of homologous or heterologous SODs. These demonstrations have been achieved in bacteria (25, 37), yeast (38, 39), *Drosophila* (40), a nematode (41), *Neurospora* (42), and even mice (43–45). The consequences of a lack of both the constitutive Fe-SOD (SOD B) and the inducible Mn-SOD (SOD A) in *E. coli* include oxygen dependent decrease of growth rate, nutritional auxotrophies, hypersensitivity toward redox cycling compounds such as paraquat and quinones, and an increase in the rate of spontaneous mutagenesis. In yeast similar problems were seen in strains lacking either the cytosolic Cu,Zn-SOD or the mitochondrial Mn-SOD. Envelope damage was made evident by the ability of osmolytes to facilitate the aerobic growth of the *sodA sodB E. coli* and to partially suppress the amino acid requirements (24).

Support for the free radical theory of senescence was provided by the shortened lifespan of *Drosophila* with a mutational defect in Cu,Zn-SOD (40). These flies were also hypersensitive toward paraquat and were sterile. A curtailed lifespan was also evident in *Caenorhabditis elegans*, which had only half the normal complement of SOD (41). Mice lacking Cu,Zn-SOD appeared normal while young but were less able to recover from axonal injury (43) and could not successfully reproduce. They also exhibited a shortened lifespan.[2] Lack of Mn-SOD imposed more serious consequences (44, 45). These animals lived only a week or two and exhibited faulty mitochondrial activities in several tissues, especially the heart.

The familial form of amyotrophic lateral sclerosis, or FALS, has been shown to be associated with defects in the gene encoding Cu,Zn-SOD (*sod* 1) (46–48). That this disease was due to a toxic gain of function of the mutated protein, rather than to a loss of SOD activity, was suggested by its genetic dominance and was demonstrated by the ability of the G93A transgene in mice to impose late onset progressive paralysis (49, 50). There is evidence for two different gains of function. One of these is a peroxidase activity (51), and the other is the ability to catalyze nitration of neurofilaments by peroxynitrite (52). Yet another novel activity of a FALS-associated Cu,Zn-SOD (H48Q) has been observed,[3] and that is a superoxide-dependent peroxidase activity. Cu,Zn-SOD has been reported to catalyze the production of HO$^\bullet$ from H_2O_2 (53), and the FALS-associated G93A mutant has been shown to be more active in this regard than the wild-type enzyme (54). However, reasons for attributing these observations to the peroxidase activity of the enzyme, rather than to the production of free HO$^\bullet$, have been delineated (6). It may be that all of these contribute to the observed pathology in FALS. Sporadic amyotrophic lateral sclerosis is clinically indistinguishable from FALS so a related mechanism might be suspected. A recent report (55) that apparent cases of sporadic amyotrophic lateral sclerosis were homozygous with respect to Asp^{90}-Ala Cu,Zn-SOD supports this notion.

Another genetic disease that may be due to faulty superoxide dismutases is progeria. Polymorphisms in the signal sequence of the gene coding for Mn-SOD have been reported (56). This led to the proposal that progeria might be due to faulty localization of Mn-SOD.

Down's syndrome (trisomy 21) is associated with a ~50% overproduction of Cu,Zn-SOD, since the gene coding for this enzyme is located on human chromosome 21. It has been suggested that this overproduction causes the symptoms of this syndrome (57). However, this seems unlikely. Thus a partial trisomy 21 with overdosages of Cu,Zn-SOD was associated with none of the usual symptoms (58). This lack of correlation of symptoms with overdosage has been replicated (59).

Varieties of Superoxide Dismutase

The SOD family has been growing. The eukaryotic cytosolic Cu,Zn-SOD and the mitochondrial Mn-SOD are now joined by an extracellular Cu,Zn-SOD, which is referred to as EC-SOD. The human enzyme is a homotetrameric protein with a M_r = 135,000. It shows some sequence homology to the cytosolic Cu,Zn-SOD but is designed for function in the extracellular spaces and is secreted by the cells that produce it. It is glycosylated and exhibits affinity for sulfated polysaccharides, such as heparin or heparan sulfate. Thus, although detectable in blood plasma, most of it exists bound onto the extracellular matrix (60–62). Its location positions EC-SOD to intercept O_2^- released by phagocytic leukocytes and other cell types. It may be important for decreasing the very rapid reaction of O_2^- with NO (63) and thus for increasing the lifetime of NO while decreasing net production of the powerfully oxidizing peroxynitrite (64). Knockout mice lacking EC-SOD develop normally but were found more susceptible to the toxic effects of 1 atm of O_2 (65). Extracellular superoxide dismutase has been reported in a variety of organisms including plants, bacteria, *Onchocerca*, parasitic nematodes, and *Schistosoma*. In the case of *Nocardia asteroides*, the extracellular SOD has been shown to be a pathogenicity factor (66).

[2] A. Reaume, personal communication.

[3] S. I. Liochev, L. L. Chen, R. A. Hallewell, and I. Fridovich, unpublished observation.

An entirely new SOD, with nickel at its active site, has been found in *Streptomyces* (67). This is a homotetrameric enzyme whose subunit weight is 13,000. It bears no obvious sequence homology to known Mn-SODs or Fe-SODs.

Cu,Zn-SODs have been reported in a few species of bacteria and were considered unusual. However, such Cu,Zn-SODs have been described with increasing frequency (68–72), and it is now likely that they occur in most Gram-negative bacteria where they are apt to be localized to the periplasm (68, 69). The *E. coli* Cu,Zn-SOD appears to be unusual in that it is monomeric (70), rather than being dimeric as are other Cu,Zn-SODs. Null mutants in Cu,Zn-SOD have been prepared; the *Caulobacter crescentis* null exhibited increased sensitivity toward citrate and toward deficiencies in Ca(II) or Mg(II) (71), whereas the *Legionella* null showed decreased survival in stationary phase (72). Sequence analysis and comparisons make it clear that Cu,Zn-SOD arose prior to the divergence of eukaryotes and eubacteria (73). It may be that the periplasmic Cu,Zn-SOD serves as a defense against exogenous O_2^-.

SoxRS Regulon

Nothing illustrates the many faceted nature of the defense against oxidative stress more than does the SoxRS regulon. This group of coordinately regulated genes is turned on by any condition that increases O_2^- production in *E. coli* (74, 75). Its products include: Mn-SOD, to eliminate O_2^-; glucose-6-phosphate dehydrogenase, to ensure a supply of NADPH; endonuclease IV, to repair oxidatively damaged DNA; the stable fumarase *c*, to replace the O_2^--sensitive fumarases *a* and *b*; ferredoxin reductase, to reductively repair oxidatively disassembled [4Fe-4S] clusters; micF, to decrease the porosity of the outer membrane; aconitase A, to replace the aconitase inactivated by O_2^-; and others as well. SoxR serves as the redox sensor, which activates production of SoxS, which then turns on the entire regulon. SoxR contains a [2Fe-2S] cluster and is a transcriptional activator only in its oxidized state. The SoxRS regulon is itself only half of the orchestrated defense against oxidative stress. There is another independent regulon turned on in response to H_2O_2 and referred to as the oxyR regulon (76).

Much remains to be told of that which is already known and much remains to be learned. I apologize to those workers who may feel slighted because their important contributions do not appear in the list of references and to the readers whose special interests are not discussed. The impossibility of fairly treating the field and those whose work created it in a minireview may be taken as another indication of the rapid growth of knowledge in this area.

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