Oxygen: How Do We Stand It?

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Reactive oxygen species · Superoxide · Singlet oxygen · Hydrogen peroxide · Nitric oxide · Free radical · Oxidative stress · Superoxide dismutase · Superoxide assay

Abstract
The electronic structure of ground state oxygen, which is essential for the life of all aerobic organisms, makes it potentially dangerous for those organisms. Atmospheric oxygen contains two unpaired electrons with parallel spin states, which predisposes it to reduction by a univalent pathway. As a consequence, normal aerobic metabolism generates dangerous reactive intermediates of the reduction of O₂. These include superoxide radical (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical (HO·). These reactive oxygen species and others that they can engender can damage all cellular macromolecules and unless opposed by cellular defenses, would make aerobic life impossible. Such defenses include superoxide dismutases, catalases, and peroxidases, enzymes that decrease the concentration of the reactive oxygen species that are their substrates, and others that repair or recycle oxidatively damaged macromolecules. Any factor that stimulates reactive oxygen species production or suppresses the antioxidant systems would inevitably cause cell damage. The role of such oxidative damage in various diseases is well documented. In vivo detection of O₂⁻ and other reactive oxygen species is however hampered by the lack of easy, specific, and sensitive analytical methods. Potential artifacts and limitations of the most common detection methods currently in use are briefly discussed.

The Basis of Oxygen’s Peculiarity

The molecular oxygen that we refer to as the ‘breath of life’ is actually a very peculiar gas. It is paramagnetic in the same way that iron is paramagnetic. If oxygen was a solid rather than a gas, we could observe it being attracted to a magnet. Yet it does respond to a magnetic field and that property is routinely exploited in oximeters that measure the oxygen content of a gas mixture.

Paramagnetism denotes unpaired electrons. Thus an electron has an associated magnetic field whose orientation depends on its spin. Two electrons that occupy the same orbital have opposite spins and so their magnetic fields are oppositely oriented and thus cancel each other. Most stable molecules have all of their electrons as such spin opposed pairs and therefore are not paramagnetic. Now let us return to oxygen whose paramagnetism denotes two unpaired electrons. The Pauli exclusion principle teaches that two electrons cannot occupy the same orbital unless their spins are opposed and, in keeping
with this principle, each of the two unpaired electrons in
oxygen occupies a separate orbital. It further needs to be
stated that these two unpaired electrons have the same
spin direction or spin state (fig. 1).

To illustrate the importance of these two parallel spin
states, try to consider adding a spin opposed pair of elec-
trons from some molecule to oxygen. One of the electrons
in the pair that happens to have a spin state opposite to
that of the unpaired electrons in oxygen could happily
join with one of them, thus creating a stable spin opposed
pair. But the other of the electrons seeking to associate
with the oxygen would necessarily have a spin that is par-
allel to that of the remaining unpaired electron in oxygen
and so could not pair with it.

The Spin Restriction

Spin restriction is the barrier to the reaction of oxygen
with all nonparamagnetic molecules. Now it is possible to
put enough energy into oxygen to elevate one of its parallel
spinning electrons to a higher orbital and in the process
to invert its spin. Such an excited state of oxygen is re-
flected to as singlet oxygen, and for singlet oxygen the spin
restriction has been eliminated and it is much more reac-
tive than is ground state oxygen (fig. 1). The energy inher-
ent in visible light is enough to convert ground state oxygen
into singlet oxygen, but oxygen does not absorb visible
light. Dyes such as methylene blue or rose bengal do ab-
sorb visible light and then, upon collision with oxygen,
can transfer the energy from that light to oxygen. That is
one basis of photosensitized oxidations. An illustration of

the power of photosensitized oxidation is provided by the
observation that NADH is not rapidly oxidized by ground
state oxygen, but in the presence of methylene blue plus
light it is. That is also an illustration of the spin restriction
in limiting the reactivity of ground state oxygen.

There is a way that the spin restriction can be circum-
vented and that is by adding the electrons to oxygen one
at a time. This works because electronic spin can be in-
verted by interacting with nuclear spin, but it is a rela-
tively slow process operating on a time scale of 10 ns.

Since the lifetime of collisional complexes is only in the
range of 0.00001 ns, spin inversion is not likely to occur
during the lifetime of collisional complexes. But there is
lots of time between collisions of potential reactants for
spin inversion to happen. That is the basis of the univa-
 lent pathway of oxygen reduction. Thus the electrons are
added to oxygen one at a time at a rate that allows elec-
tronic spin inversions to occur between collisional events.

The Univalent Pathway

The univalent pathway of oxygen reduction requires
that intermediates of oxygen reduction be generated
(fig. 2). Thus the complete reduction of oxygen to water
requires that four electrons and four protons be added to
the oxygen molecule, yielding two molecules of water.
Adding the first electron produces O$_2^-$; adding the sec-
ond electron plus two protons yields H$_2$O$_2$; adding the
third electron gives HO$^+$ plus OH$^-$; and finally adding the
fourth electron plus two more protons produces two wa-
ter molecules. These intermediates of oxygen reduction
are reactive and can damage biological molecules. In-
deed, were there not defenses against them, these inter-
mediates would make aerobic life unsustainable.

The Intermediates of Oxygen Reduction

The first intermediate encountered on the univalent
pathway is superoxide (O$_2^-$). It is the conjugate base of the
weak acid, the hydroperoxyl radical (HO$_2$). Its pKa is 4.8,
so at neutrality it is 99% ionized. O$_2^-$/HO$_2^-$ is not stable
in protic solvents and dismutates spontaneously into H$_2$O$_2$
plus O$_2$ with rate constants that depend on pH [1]. Thus
at low pH, where it exists primarily as HO$_2^-$, the rate con-
stant is close to $10^5$ M$^{-1}$ s$^{-1}$. At pH 4.8, where it exists as
half HO$_2^-$ and half O$_2^-$, the rate constant is $10^8$ M$^{-1}$ s$^{-1}$.
At high pH it is mostly O$_2^-$ and then the rate constant is
essentially zero. The reason for this is that the electro-

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Fig. 1. Electronic configurations energy levels of ground state and
singlet molecular oxygen.
static repulsion between two \( \text{O}_2^- \) anions prevents close approach and hence prevents the electron transfer between them, which is the basis of the dismutation.

The second intermediate, \( \text{H}_2\text{O}_2 \), is not a free radical and is relatively stable. However, it can be univalently reduced by metal cations such as ferrous and cuprous to yield the hydroxyl radical (\( \text{HO}^- \)) plus hydroxide ion (\( \text{OH}^- \)). This is known as the Fenton reaction and the \( \text{HO}^- \) it generates is an extraordinarily powerful oxidant. It is also the third intermediate encountered on the univalent pathway of \( \text{O}_2 \) reduction. All of these intermediates are able to damage the components of cells in ways that we will explore next.

**How \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \), and \( \text{HO}^- \) Cause Damage**

The superoxide radical can act either as a reductant or an oxidant. Thus its ability to reduce cytochrome c was important in its discovery as a product of the aerobic xanthine oxidase reaction and is currently the basis of an assay used to measure the activity of superoxide dismutases. In the reducing environment of cell cytosol, \( \text{O}_2^- \) is more likely to act as an oxidant, and one important target for its action as an oxidant is the iron-sulfur clusters (4Fe4S) of dehydratases, such as that found in aconitase [2]. Superoxide can oxidize these clusters and so inactivate this family of enzymes. This blocks the citric acid cycle that is so essential for the aerobic metabolism of cells, and it has an additional far-reaching consequence. Thus, a (4Fe4S) cluster that has been univalently oxidized by \( \text{O}_2^- \) is unstable and decomposes, releasing free iron that can then participate in the Fenton reaction that generates the powerful oxidant, \( \text{HO}^- \) [3], which can oxidize virtually any biological molecule. Hydroxyl radical is so reactive that it will react with whatever is close by, which would lead one to suppose that it would be consumed by the plethora of relatively unimportant metabolic intermediates. However, when we consider what a cationic metal such as \( \text{Fe}^{2+} \) would bind to, polyanionic species such as DNA, RNA, and cell membranes come immediately to mind. Hence, they would be the preferential targets for the \( \text{HO}^- \) generated by the Fenton reaction. Another point to be considered is that \( \text{HO}^- \) is a univalent oxidant. Hence it would produce free radicals derived from its targets, and that opens the door to the propagation of chain reactions that would amplify the damage caused by one \( \text{HO}^- \). This is especially the case with biological membranes, DNA, RNA and macromolecules in general (for details see [4]).

**Defensive Strategies**

The first obvious defense is avoidance. Thus, there are enzymes that reduce oxygen divalently to \( \text{H}_2\text{O}_2 \) and even tetravalently to water without releasing intermediates such as \( \text{O}_2^- \). D-amino oxidase is in the first category and cytochrome c oxidase is in the second. Yet, because of the spin restriction described above, virtually all auto-oxidations and even some enzyme-catalyzed reactions do generate \( \text{O}_2^- \). In respiring cells only about 0.1% of the oxygen consumed is released as \( \text{O}_2^- \) [5]. Yet given the large amounts of oxygen utilized in respiration, even so small a fraction as 1/1,000 creates \( \text{O}_2^- \) at intolerable rates. Hence defenses are essential to limit and to repair the damage that \( \text{O}_2^- \) and its progeny \( \text{H}_2\text{O}_2 \) and \( \text{HO}^- \) would otherwise do. A detailed description of the best studied defense mechanisms and their regulation can be found in a recent review [6].

**Superoxide Dismutases**

The primary defense is provided by superoxide dismutases (SODs) that catalyze the conversion of \( \text{O}_2^- \) into \( \text{H}_2\text{O}_2 \) plus oxygen. A dismutation is a reaction that converts two identical substrate molecules into two different products; hence the name. There are several different SODs. One member of this family is the Cu,ZnSODs [7]. These enzymes are found in the eukaryotic cytosol, nuclei, the intermembrane space of mitochondria, in the periplasm of Gram-negative bacteria and in the extracellular spaces of multicellular eukaryotes. This wide distribution indicates that \( \text{O}_2^- \) is encountered in all of these places and presents a threat in all of them.
Mechanism

Cu and Zn are present in an imidazole bridged bimetallic structure at the active sites of this homodimeric protein. Copper is the catalytic metal and is reduced from Cu²⁺ to Cu⁺ during the first interaction with O₂⁻, and then is reoxidized by the next O₂⁻ encountered [8]. Zn plays a structural role and also holds the bridging imidazole in place during the catalytic cycle. This is an extraordinarily active enzyme whose interactions with O₂⁻ occur with a rate constant of 2 \times 10⁹ M⁻¹ s⁻¹ 25°C [9]. At such a diffusion-limited rate, proton conduction would be rate-limiting in the absence of some special facilitating mechanism. The bridging imidazole provides that facilitated proton conduction. Thus, when the Cu²⁺ is reduced in the first step of the catalytic cycle, it re-leases the bond to imidazolate, which then binds a proton from water. In the second step of the catalytic cycle, during which the bond to Cu²⁺ is re-established, that proton is released to form the leaving HO₂⁻ and it gets the second proton from the bulk water to form H₂O₂. There is much homology among all the Cu,ZnSODs, particularly with regard to the active site structure. Nevertheless, there are differences. Thus, some of the bacterial periplasmic Cu,ZnSODs are monomeric [10, 11], while the extracellular SOD of mammals is tetrameric and is also glycosylated [12].

The MnSODs and the FeSODs

These SODs exhibit a great deal of homology, but are usually specific for the metal that constitutes the active site [13–15]. Thus the FeSOD found in Escherichia coli can be stripped of its metal and the resultant apoenzyme can be reconstituted with either Fe or Mn. If reconstituted with Fe, all of the activity is restored, but if reconstituted with Mn, it remains inactive [16]. In fact, the two metals can be shown to compete for binding to the active site. Some facultative anaerobes do contain a single SOD that can be active with either Fe or Mn at the active site [17]. These are called cambialistic SODs and when the organism is grown anaerobically, Fe is the metal inserted, but when grown aerobically, Mn is inserted. MnSOD is not only found in bacteria, but also in the matrix of mitochondria. Indeed, the parallel between Gram-negative bacteria and mitochondria is striking and can be taken as support for the endosymbiotic origin of these organelles [18]. Thus both E. coli and mitochondria have MnSOD in the cytosol and matrix respectively, and both have Cu,ZnSOD in the periplasm and intermembrane space, respectively (for details see [19]).

One might wonder why E. coli should be able to produce both a FeSOD and a MnSOD. The FeSOD is made at all times, whether the environment is aerobic or anaerobic, while the MnSOD is made under aerobic growth. Facultative microorganisms, such as E. coli, must face the possibility of sudden transfer from anaerobic to aerobic conditions. Hence the FeSOD can be viewed as a standby defense available at all times, while the induction of MnSOD provides for fine tuning of the level of defense to the level of threat. Fe²⁺ is stable and soluble anaerobically, but is prone to oxidation to Fe³⁺ aerobically, and Fe³⁺ forms insoluble hydroxide and phosphate salts. Mn salts, in contrast, remain soluble and thus available under both conditions. Hence it makes good sense that E. coli should make FeSOD at all times even anaerobically and induce the production of MnSOD aerobically. The same reasoning applies to the cambialistic SOD made by Propionibacterium shermanii.

Why So Many Types of SOD?

In addition to the Cu,ZnSODs, MnSODs, and FeSODs already mentioned there is a NiSOD found in Streptomyces [20]. All of these enzymes are comparably active. It is helpful to consider that there must have been a variety of organisms living in a variety of habitats that evolved during the anaerobic phase of this planet’s history. Then, with the advent of true photosynthesis in the cyanobacteria, oxygen accumulated in the biosphere and that variety of anaerobes had to evolve SODs or perish. Different SODs evolved, depending on which metal was most available in the environment, and those different SODs are still here [21].

Assays for SOD Activity

Superoxide is not stable in protic solvents such as water, so it is not feasible, except by pulse radiolysis, to assay SOD by following its effect on the rate of consumption of its substrate. One way around this impasse is to have a reaction that produces a constant flux of O₂⁻ and to allow that O₂⁻ to react with some chromogenic substrate. In such a reaction system, SOD will compete with the chromogenic substrate for the flux of O₂⁻ and thus inhibit the rate of color change. The activity of the SOD can then be derived from its inhibition of the color change. In the classical assay, the xanthine oxidase reaction provides the flux of O₂⁻ and cytochrome c acts as the chromogen [22]. The sensitivity of this assay system depends upon the rate of production of O₂⁻ and on the concentration of cytochrome. Obviously, anything that inhibits xanthine oxidase will decrease the rate of reduction of the cytochrome.
and thus appear to have SOD activity. Likewise, any compound that competes with the cytochrome c for O$_2^-$ will result in an apparent decrease in [SOD] or look like an inhibitor of SOD [23]. A control is needed to establish that the material being tested does not directly inhibit xanthine oxidase.

In other assays, the source of the flux of O$_2^-$ and the chromogen can be one and the same. Thus a compound that oxidizes spontaneously and so generates O$_2^-$ that propagates that oxidation to a final chromogen provides the basis of such an assay. Pyrogallol and epinephrine auto-oxidations are such O$_2^-$ that oxidizes spontaneously and so generates O$_2^-$ as well as detect it. For example, the enzyme glucose oxidase reduces oxygen to hydrogen peroxide. It

Superoxide Assays Applied to Cells and Tissues

Investigators have devoutly wished for assays that would measure O$_2^-$ within cells and tissues. This is intrinsically difficult. Thus, it would require a cell-permeable compound that was modified specifically by O$_2^-$ to a stable product that could be detected spectrophotometrically or fluorimetrically. Furthermore, this compound would have to compete favorably with cellular SODs for the flux of O$_2^-$.

Oxidative Stress and Medicine

The realization that the reactive and damaging intermediates of oxygen reduction are routinely made in cells led to the awareness that they may be involved in a variety of disease processes. This idea gained considerable traction from the finding that activated leukocytes undergo a respiratory burst that is accompanied by the formation of a large quantity of O$_2^-$ [32], and that genetic defects in the ability of the leukocytes to exhibit this respiratory burst are the basis of chronic granulomatous disease as well as detect it. For example, the enzyme glucose oxidase reduces oxygen to hydrogen peroxide. It does not generate O$_2^-$ but it does reduce NBT to a tetrazazolium that is not reduced to an auto-oxidizable intermediate provides a way to circumvent this artifact. The tetrazolium XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2-tetrazolium 5-carboxanilide) has these properties and has been used to good advantage [28, 29].

Direct XTT reduction by NADPH:XTT reductases, however, makes the XTT assay unreliable in vivo [30].

Orchestrated Defenses

The ubiquity of oxidative stress has called forth the evolution of coordinately regulated cohorts of enzymes dedicated to defending cells against these stresses and to repairing the damage due to oxidation. In E. coli, two
such orchestrated defenses against oxidative damage have been described. One is called the soxRS regulon and the other is known as the oxyR regulon [19]. The soxRS regulon depends on the oxidation state of an iron-sulfur cluster in the SoxR protein [39]. When this cluster is oxidized, SoxR binds to and activates the operator of SoxS causing SoxS to be made; in turn, SoxS activates the genes for all the members of the regulon [40]. Over tens of genes are members of the soxRS regulon, and that indicates how important it is to defend against oxidative stress. One member of this regulon is the MnSOD that scavenges O2−; another is endonuclease 4 that helps repair oxidative damage to DNA [41]. Dehydratases that contain 4Fe4S clusters, such as fumarases A and B and aconitase B, are particularly susceptible to oxidation by O2− [42, 43], and the regulon includes fumarase C and aconitase A that are less susceptible to such oxidative inactivation [44]. The environment abounds in compounds that can mediate the oxidation of NAD(P)H with the concomitant formation of O2−. One member of the soxRS regulon serves to diminish the permeability of the cell envelope to such compounds [45]. This small sampling of the functions of regulon serves to illustrate the variety of things that contribute to the defense against oxidative stress in E. coli. OxyR is regulated by the oxidation of a thiol group on the OxyR protein [46, 47]. This thiol can be oxidized by H2O2 to a sulfenic acid that then reacts with a nearby thiol to generate a disulfide bond [48]. This changes the conformation of OxyR so that it binds to and activates the genes coding for the members of this regulon that include a catalase, glutathione peroxidase, and an alkylhydroperoxidase, among others [19].

Other Radicals

Nitric oxide (NO−) is produced in many organisms and serves as a signaling molecule. NO−, like O2− is a free radical and radical-radical reactions are fast. So it is no surprise that O2− and NO− react with a diffusion limited rate constant to yield peroxynitrite, and that is a strong oxidant in its own right and can also homolysse to give two reactive radicals, i.e. NO2 and HO· [49]. This leads to the nitration of tyrosine residues in proteins and polyunsaturated fatty acids in phospholipids, and such modified tyrosines and fatty acids can be detected in inflamed tissues [50, 51]. Another strongly oxidizing radical likely to be encountered in living things is the carbonate radical [52].

Epilogue

We now realize that the oxygen that is now so abundant in the earth’s atmosphere, and is so essential for the life of aerobes such as we, is at the same time the progenitor of threatening reactive species. The study of the manifold effects of these reactive oxygen species and the defenses that allow aerobes to harvest the benefits of oxygen while surviving its onslights is still in its infancy. Pursuit of such studies will expand our understanding and provide solutions to currently intractable problems. As the Count de La Rochefoucauld once said, ‘Knowledge is the only way out of the cages of life’.

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