HHS Public Access

Author manuscript

Trends Microbiol. Author manuscript; available in PMC 2022 May 01.

Published in final edited form as:

Trends Microbiol. 2021 May ; 29(5): 428–440. doi:10.1016/j.tim.2020.10.001.

How Microbes Evolved to Tolerate Oxygen

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Abstract

Ancient microbes invented biochemical mechanisms and assembled core metabolic pathways on an anoxic Earth. Molecular oxygen appeared far later, forcing microbes to devise layers of defensive tactics that fend off the destructive actions of both reactive oxygen species (ROS) and oxygen itself. Recent work has pinpointed the enzymes that ROS attack, plus an array of clever protective strategies that abet the well known scavenging systems. Oxygen also directly damages the low-potential metal centers and radical-based mechanisms that optimize anaerobic metabolism; therefore, committed anaerobes have evolved customized tactics that defend these various enzymes from occasional oxygen exposure. Thus a more comprehensive, detailed, and surprising view of oxygen toxicity is coming into view.

Oxidative Stress: Then and Now

Thirty years ago Irwin Fridovich authored a paper in which he outlined a new view of how oxidative stress shaped the evolution of life on Earth [1]. After years of uncertainty and controversy, researchers had proved that superoxide (see Glossary) stress is real and that superoxide dismutase (SOD) is a dedicated defense against it. The catalytic mechanisms and evolutionary history of SOD had been clarified. Shortly before publication, researchers had finally identified enzymes that superoxide can disable, although the underlying molecular mechanisms were still unclear.

Fridovich's paper also repeated some ideas that were popular at the time but that since have turned out to be incomplete or incorrect. For example, it was widely suspected that anaerobes had fled oxic environments because they failed to acquire scavenging enzymes, that superoxide supplied electrons to drive the Fenton reaction, and that intracellular respiration could shield cells from oxygen.

Dr Fridovich ended his paper by noting 'Novel findings often lead to unexpected clarifications. A number of surprises has already turned up in the course of this work. I think there will be more.'. In this review we highlight some of the surprises that turned up – and some of the aspects that continue to perplex.

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The Rise of Oxygen

Life started and diversified in a world devoid of oxygen – which had a profound effect upon the catalytic strategies that were available to early microbes and that have been inherited by contemporary organisms. The anoxic world had a highly reducing environment, and iron, which is most soluble in its ferrous form, was therefore relatively bioavailable [2]. Iron lies in the sweet spot of transition metals: it has a reduction potential that is appropriate for biological redox reactions, and it outperforms other transition metals as a surface catalyst by having a mid-range binding affinity that excels at substrate capture and release [3]. Therefore, iron cofactors were readily incorporated into numerous enzymes – in effect, many metabolic pathways are organized around the chemistry that iron can catalyze. Within contemporary organisms, for example, iron-cofactored enzymes catalyze all the redox transitions necessary for sulfur and nitrogen assimilation: the tricarboxylic acid (TCA) cycle and pentose phosphate pathway; all forms of respiration; the syntheses of branched-chain and aromatic compounds; ribonucleotide reduction and steps in DNA repair; the syntheses of lipoate, biotin, thiamine, quinones, and numerous other cofactors. *Escherichia coli* alone has 140 enzymes that use iron–sulfur clusters as catalytic centers [4].

This reliance upon iron-driven chemistry was destabilized by oxygenic photosynthesis, an event that upended the geology, atmosphere, and the biota of the Earth in comprehensive ways. The invention of a highly oxidizing photosystem II enabled ancient cyanobacteria to use water as an electron donor for NADPH formation [5,6] – thereby liberating these bacteria from a requirement for other environmental reductants, such as sulfur species. However, the by-product of water oxidation is molecular oxygen. The timing of the appearance of photosystem II is not settled; some researchers propose 3–3.8 billion years ago (Gya) [7–9], while others suggest that it evolved around 2.35 Gya [10,11] (Figure 1). In either case, the impact upon atmospheric and dissolved oxygen was not immediate as the nascent O_2 was chemically scavenged by the sulfur and iron species in the environment. The oxygen concentration in the Archaean was estimated to be as low as 10^{-5} of the present atmospheric level (PAL) [12], and the standard two-step model is that its concentration increased to 1–10% of the PAL at the Great Oxygenation Event (GOE) about 2.3 Gya, with a second jump to PAL about 0.5 Gya [13] (Figure 2A). An alternative suggestion is that atmospheric oxygen jumped close to the PAL soon after the GOE, followed by a steep fall to 0.01–0.1 PAL [14]. The oxygen-lean atmosphere extended for about another billion years until the second rise to PAL.

This history indicates that microbes had highly defined biochemical and metabolic plans in place long before oxygen created problems. Further, the gradual nature of oxygenation predisposed microbial evolution towards a prolonged series of layered adaptations to it; there was no abrupt crisis to force the abandonment of the core plan. Consequently, contemporary organisms feature a metabolic map that is largely unchanged from that of their ancient anaerobic forebears. This has consequences.

The Appearance of Aerobic Metabolism

Molecular oxygen is a reactive chemical, and at first blush it might seem that its immediate toxicity should have precluded the evolution of cells that perform oxygenic photosynthesis. However, the degree of oxygen exposure endured by primordial cyanobacteria was minimal. Oxygen moves rapidly across lipid bilayers – it crosses a membrane more readily than it does a water layer of the same thickness [15] – and so photosynthetically produced oxygen quickly escapes the cell rather than accumulating internally. Models predict that the excess oxygen concentration in the most primitive known cyanobacterium, *Gloeobacter violaceus*, would have been four orders of magnitude lower than in contemporary air-saturated water (0.025 μM versus 230 μM) [15]. Such concentrations would have been relatively innocuous, as photosynthetic cells can survive up to 2.3 μM intracellular O_2 without specialized defensive mechanisms [15]. Thus it is likely that oxygen stress remained insignificant for eons until cyanobacteria evolved to grow in biofilms or bacterial mats, where local oxygen levels can rise due to macroscopic diffusion barriers [15].

The most obvious impact of oxygen accumulation was the appearance of oxygen-directed respiration, an enormously exergonic form of metabolism that was an important driver, if not an essential one, in the evolution of complex life forms and eukaryotes [16,17]. Cyanobacteria were once again at the forefront of this evolutionary event: it is widely accepted that aerobic respiration also evolved in this lineage of bacteria [10], and not only once [18]. Notably, although respiration profoundly changed the overall metabolic scheme – prototrophic photosynthetic bacteria were transformed into oxidizing heterotrophs – the amount of evolution that was required at the molecular level was surprisingly small. Anaerobic electron transport chains had already employed complexes I–III so that the membrane potential could push electrons uphill from high-potential donors like sulfide to $NADP^+$; once oxygen appeared, it only required the plug-in of a novel cytochrome oxidase for the electron transport chain to reverse its course, producing a membrane potential rather than consuming one (Figure 3). Thus the appearance of O_2-directed respiration required only a modest evolutionary step – and did not involve a gross redesign of cells. Accordingly, dedicated aerobes that exploit oxygen as an electron acceptor have retained many ancestral biochemical features that are now problematic in oxic environments.

It may seem odd that molecular oxygen is both a boon and a threat to bacteria. Interestingly, this evolutionary pattern appears to have recurred in the evolution of membrane-bound peroxidases which similarly plug into the respiratory chain and enable bacteria to use another potentially toxic oxidant, H_2O_2, as an electron acceptor [19].

The First Problem: A Change in Bioavailable Metals

Both the geological record and the modeling of ocean chemistry suggest that oxygenation triggered two shifts in the levels of dissolved metals: first between the Archaean and Proterozoic oceans, and later between the end of the Proterozoic and the development of the modern ocean [2,20] (Figure 2B). Changes in manganese, cobalt, molybdenum, and nickel concentrations likely had impacts upon their recruitment into specialized enzymes; but of particular interest to this account is the four-order-of-magnitude drop in iron levels due to

the insolubility of the oxidized ferric form, and subsequently a large increase in copper levels when the oxidation of sulfides released it from mineral precipitates. The depletion of iron put microbes in an untenable position, given their reliance upon it for so many enzymes – yet, because it was used so broadly, there was not a strong selection for a particular enzyme to abandon iron as a cofactor: a single change would have had a minimal impact upon the total iron demand of the organism. Thus, bacteria did not evolve towards iron-free metabolism.

Indeed, modern-day *E. coli* still contains nearly 1 mM iron, almost all of it locked into its enzymes [21]. Like many other microbes, *E. coli* copes with occasions of iron deficiency in several make-shift ways: by secreting siderophores to solubilize environmental iron, by withdrawing ferric oxide deposits from storage proteins, by shifting to metabolic strategies that require fewer iron-dependent enzymes, and by importing manganese to substitute for iron in mononuclear enzymes [22,23]. These adjustments are merely palliative, and iron scarcity in an environment can substantially shift the density and identity of the local microbial community. The ongoing struggle of microbes for iron is also manifested by the phenomenon of nutritional immunity: the tug-of-war between the host and the pathogen for this element has resulted in the evolution of shrewd strategies on both sides [24].

By contrast, oxygenation caused the solubilization of copper at the end of the Proterozoic era, ca. 0.5 Gya, and this triggered the appearance of copper enzymes relatively late in evolution. This metal shares some of the useful redox behaviors of iron; but because of its affinity for reduced sulfur species, copper enzymes are used exclusively in oxic environments and are localized in nonreducing cell compartments and the extracellular space. The copper enzymes of bacteria are predominantly found in the periplasm or attached to the cell envelope. It is notable that the cytoplasm of bacteria – which is rich in sulfur species and maintains iron in its reduced form – preserves the utility of iron by retaining the iron/sulfur redox poise of the ancient Earth on which life evolved.

The Second Problem: The Toxicity of ROS

At the time of the Fridovich paper, 30 years ago, the toxicity of ROS had become broadly accepted – and its linkage to iron biochemistry was becoming evident. Genetic studies had shown that SODs were essential for *E. coli* to thrive in oxic culture media [25], and inducible regulons had been discovered that provided resistance to severe superoxide and hydrogen peroxide stress [26–28]. Yet little was known about the intracellular sources and targets of these oxidants, nor about other cellular strategies that prevent or reduce oxidative damage.

We now know that ROS are formed continuously inside aerobic cells as molecular oxygen oxidizes the univalent cofactors of their redox enzymes. The molecular details and rates of these adventitious reactions have been described in detail [29]. Researchers have identified multiple targets this ROS can then damage, the most important of which are non-redox enzymes that use iron cofactors to bind and activate their substrates (Figure 4). Both superoxide and hydrogen peroxide oxidize and release the catalytic iron atom from mononuclear iron enzymes (including epimerases, dehydrogenases, deformylases, and

deaminases) [30,31] and the solvent-exposed iron of [4Fe-4S] clusters of dehydratases [32–34]. The consequent inactivation of these enzymes causes multiple pathway failures in SOD- and catalase/peroxidase-deficient cells, with the net effect of blocking growth. Reactions of hydrogen peroxide with the pool of loose iron – which exists to metallate iron-dependent enzymes – also produces hydroxyl radicals, and their oxidation of DNA is a primary source of mutagenesis [35]. Thus, univalent redox chemistry and iron catalysts were the tactics that enabled life to evolve and thrive in the anaerobic world – but, as oxygen levels rose, their continued use predisposed microbes to both ROS formation and its toxicity.

The Evolution of Defenses against ROS: Scavenging Systems

The growing oxidative stress necessitated the evolution of antioxidant systems. The earliest forms of these systems may have been small molecules and metals [36] such as manganese [37], sulfur complexes [38], and carotenoids [39]; some of these remain important parts of the antioxidant systems in prokaryotes and eukaryotes. Stronger defenses arose with the evolution of enzymes that directly degrade O_2^- and H_2O_2.

The evolutionary timeline of antioxidant enzymes, like that of oxygenic photosynthesis, is controversial. While some researchers believe that they evolved during the GOE [40,41], recent works have dated the origin of the ROS-scavenging enzymes to 3.5–4.1 Gya [42,43] (Figure 1). This estimate coincides with the earliest reports of oxygenic photosynthesis (at ~3.7 Gya), suggesting that oxygen, and hence ROS, may have accumulated in microenvironments of the early oxygen-producing organisms and thereby driven the evolution of antioxidant systems [14,44–46]. The initial scavengers may have been the superoxide reductases and peroxidases that still prevail in reducing environments; the disproportionating enzymes – SOD and catalases – may have arisen later as a scavenging strategy in cells that operate at higher potentials. Analyses generally support the notion that the manganese- and iron-dependent superoxide dismutases predate the copper-dependent SODs – which conforms with the geological abundances of these metals [43].

Detailed phylogenetic studies of scavenging enzymes might clarify the evolutionary byplay between evolving life forms and oxidative stress. That approach is complicated, however, by the nature of these defenses. Most antioxidant enzymes are encoded by single genes that can dramatically increase the fitness of an ROS-stressed organism; these features made their structural genes suitable for horizontal gene transfers and duplications [47,48], which muddy the phylogenetic record.

Indeed, it can be difficult to correctly identify antioxidant enzymes in the genomic data or even in biochemical experiments. These enzymes have simple structures and they operate based on electron-transfer principles that can be mimicked by adventitious events – as manifested by the chance degradation of superoxide by mononuclear iron enzymes [30] and of hydrogen peroxide by cytochrome *bd* oxidases [49]. The simplicity of these reactions has repeatedly created uncertainty over whether the *in vitro* ROS-degrading activity of an enzyme represents its actual *in vivo* function. Indeed, it was this type of ambiguity that spawned the original heated debate over the physiological role of SOD [50]. As this example showed, careful *in vivo* studies are necessary to correctly identify authentic scavengers.

Because atmospheric oxygen increased only gradually over the past 2.5 billion years, there was ample time for the refinement and dispersal of defensive systems. The result is that cells acquired layers of protection that extend beyond simple scavenging systems.

The Evolution of Defenses against ROS: Beyond Scavenging

In addition to scavenging enzymes, cells acquired other mechanisms to prevent or relieve ROS stress. Evidence suggests that the electronic structures of some redox enzymes have been adjusted in order to minimize ROS formation in microbes that frequently dwell in oxic environments. The enzyme fumarate reductase provides a route for anaerobic respiration – and its aerobic paralog, succinate dehydrogenase, drives the TCA cycle. A key difference between the two enzyme forms is that fumarate reductase leaks electrons to oxygen, whereas succinate dehydrogenase does not [51,52]; the difference is a result of subtle shifts in cofactor redox potentials, thus pulling electrons away from the auto-oxidizable flavin. Measurements of intracellular ROS formation are in their infancy, but early data suggest that aerobes generate fewer ROS in oxic environments than do anaerobes [53].

Other adaptations protect specific ROS targets. In many bacteria, the [4Fe-4S] cofactors of dehydratases and the Fe(II) cofactors of a variety of enzymes are important targets of O_2^- and H_2O_2 [32–34]. Microbes have evolved a variety of strategies to cope with this hazard (Box 1), including the replacement of iron with manganese in mononuclear enzymes. Lactic acid bacteria – which generate H_2O_2 as a routine by-product of their aerobic metabolism – routinely populate these enzymes with manganese [54]. After the GOE, manganese was more bioavailable than iron, and it is striking that it was recruited into mangano forms of SOD, catalase, and ribonucleotide reductase. The gradual displacement of iron by manganese may have been a broad feature of evolution after atmospheric oxygenation [37,55].

The third known threat of ROS stems from the reaction of H_2O_2 with the cytoplasmic pool of loose ferrous iron. This event generates hydroxyl radicals – which go on to oxidize a range of biomolecules. Microbes lack polyunsaturated fatty acids and are thereby spared the lipid peroxidation that plagues eukarya [56]; however, their DNA is damaged, and it seems likely that this threat imposed pressure for the evolution of DNA-repair enzymes [57]. Bacterial mutants that lack both recombinational and excision-repair mechanisms can survive in anoxic environments but are killed by oxygenation [58,59] Bacteria also evolved several tactics to minimize this problem by limiting the size of their intracellular iron pools. Various transcription factors shut down iron acquisition once the pools are sufficient [60,61], and in aerobic cells extra iron is stored as ferric hydroxide crystals in ferritins, a process that uses oxygen as a co-reactant [23]. During H_2O_2 stress an additional miniferritin, Dps, is induced and uses H_2O_2 itself as the co-reactant; the effect is to shrink the loose-iron pool and thereby avert Fenton chemistry [35,62]. Notably, these diverse defenses – scavenging enzymes, suppressed ROS formation, cluster repair, modified dehydratases, manganese import, DNA repair enzymes, ferritins and Dps – are all found in the same cells, demonstrating that nature evolved layers of tactics to suppress ROS stress.

The Toxicity of Molecular Oxygen and Commitment to Anaerobiosis

The early studies of oxidative stress were conducted in organisms (*E. coli*, and *Saccharomyces*) and human cells that were familiar to biologists. But these are also organisms that occupy aerobic niches and by definition have successfully evolved to cope with oxygen. Perhaps a better indication of the stress that was imposed by ancient oxygenation is acquired through study of contemporary obligate anaerobes. In recent years the cultivation and biochemistry of anaerobes has developed far beyond its status 30 years ago, and it has become clear that ROS were not the sole, or perhaps even primary, oxygen-triggered stress.

Obligate anaerobes do have ROS-scavenging systems and repair mechanisms which enable them to survive – but not grow – during episodic exposures to oxygen. Modern anaerobes experience oxidative stress, for example, when they transit between hosts, when rainwater penetrates into the anoxic zones of the soil [63], and when gut microbes encounter the gradient of oxygen from the epithelium into the intestinal lumen [64]. Their reliance upon a mixture of oxidant-sensitive and oxidant-resistant components has allowed various microbes to push the borders between the oxic and anoxic world in one way or another [65]. Indeed, life forms exhibit a continuous spectrum of oxygen tolerance, from obligate anaerobiosis to obligate aerobiosis, at the extremes of which the former group ceases growth in the presence of oxygen, and the latter in its absence.

We have learned that obligate anaerobes cannot tolerate full aeration because oxygen poisons their core strategies of energy production [66–70] These strategies rely upon low-potential and free-radical biochemistry that can drive reactions on highly reduced substrates. Such reactions allow anaerobes to excel in anaerobic habitats; in the gut, for example, obligately anaerobic *Bacteroides* species outnumber facultative (oxygen-tolerant) anaerobes by four orders of magnitude [71]. The same biochemical features, however, are the ones that render these bacteria susceptible to oxygenation (Figure 4). Molecular oxygen directly adducts enzyme radicals and fractures the enzymes that depend upon them. It oxidizes and inactivates low-potential enzymic metal centers. It also drives the production of copious ROS, perhaps through those metal-center oxidations [53]. Therefore, even if anaerobes were to allocate a large portion of their resources to scavenging ROS, they would still fail to prevent the disabling injuries that molecular oxygen produces directly. As a consequence, evolution created the dichotomy between anaerobes and aerobes, with anaerobes retaining the features that make them competitive in anoxic habitats at the cost of foreclosing growth upon occasional exposure to oxygen. It is likely that, in their occasional conflict with oxygen, contemporary anaerobes recapitulate the crisis that was experienced by ancient microorganisms at the time of the GOE.

The Evolution of Defenses against Molecular Oxygen

There is no zero in biology, so even anaerobes in hypoxic habitats require responses that boost their ability to tolerate trace oxygen. As biochemists have dissected the catalytic mechanisms of oxygen-sensitive enzymes they have discovered wildly diverse adaptations that improve oxygen resistance [72]. Nitrogenases, for example, are ancient oxygen-sensitive enzymes that appeared in methanogens long before the GOE; however, these

enzymes have a physiological role suitable for facultative and aerobic microbes as well [73]. Some of the latter species sustain nitrogenase function by keeping a very low internal oxygen concentration – which they do by using biofilms to create a hypoxic local environment or by buffering oxygen with hemoglobin analogs [74]. The Shethna protein provides another route of protection, occluding (and inhibiting) the vulnerable metal centers of the enzyme for the duration of oxygen exposure [75]. Like nitrogenases, a group of hydrogenases have also evolved a unique approach to oxygen toxicity. The hydrogenase reaction is intrinsically reversible, and its physiological direction depends upon its role in metabolism. Hydrogen-evolving hydrogenases are useful only in anaerobes, and their di-iron sites are vulnerable to oxidation [76]; yet hydrogen-consuming hydrogenases – which productively operate under oxic conditions – have evolved a more-resistant Ni–Fe site [77], and some of these enzymes coordinate a proximal iron–sulfur cluster in an unconventional geometry that circumvents local radical formation and prevents peptide damage [78].

Specialized enzyme-repair systems have also arisen. The B_{12} cofactors are occasionally oxidized; a dedicated chaperone extracts the damaged cofactor, replaces it with a new one, and thereby revives enzyme activity [79]. When oxygen attacks the catalytic glycyl radical of pyruvate:formate lyase (PFL), ultimately cleaving the polypeptide chain, facultative anaerobes respond by inducing a small protein that displaces the ruined fragment and permits the regeneration of the radical [80]. This arrangement speeds the recovery of PFL activity after these bacteria experience brief oxygen exposure.

The take-home message is that while the presence of oxygen is typically black-or-white in the laboratory, in natural habitats oxygen levels can rise and fall situationally, and microbes had to devise methods to cope. The examples cited here were mostly discovered by chance, and so – intriguingly – they likely constitute just the tip of the iceberg.

Has Evolution Solved Oxygen Toxicity?

No, it has not. Microbes manifest a range of protective strategies that is wide and clever – but a universal observation is that organisms still cannot tolerate oxygen levels much above those of their native habitats. This persistent sensitivity suggests that, even in their normal environments, they likely suffer some degree of injury – and that, if oxidative stress were enhanced, growth would suffer. The latter principle is exploited by the interspecies warfare that pervades competitive environments. Plants and bacteria secrete redox-cycling compounds that generate toxic doses of O_2^- inside target organisms [81,82], and lactic acid bacteria shower their cohabitants with hydrogen peroxide [83,84]. Amoebae, plants, and higher animals use NADPH oxidases to generate enough ROS to kill invading microbes [85,86]. Successful pathogens push back by disabling the oxidases and augmenting their own scavenging systems [87,88]. Thus, the core vulnerabilities, descended from ancient anaerobes, remain at risk; evolution has merely succeeded in shifting the tipping point.

Concluding Remarks

The generation of oxygen was a calamitous event that redirected evolution and that continues to structure every microbial community on the planet. In the past 30 years a much

more detailed view of oxidative stress has emerged. Researchers have pinpointed the intracellular targets that O_2^- and H_2O_2 attack, and they have revealed the mechanisms by which damage occurs. New defensive tactics have come to light. The phenomenon of obligate anaerobiosis is now attributed to both molecular oxygen and its reactive species. We remain, however, in the dark on several important points (see Outstanding Questions). It is not clear why oxygen generates more ROS in some organisms than in others. The molecular details of enzyme damage by oxygen are still being investigated. We do not know how anaerobes manage to resume their metabolism after episodes of exposure to oxygen. These questions – and unexpected surprises – provide fodder for the next 30 years.

Acknowledgments

This review is dedicated to the memory of Irwin Fridovich. The work by the authors is supported by National Institutes of Health (NIH) award GM49640.

Glossary

B_{12}
(or cobalamin) a coenzyme that produces a shielded adenosyl radical important in radical-based reactions.

Fenton reaction
electron transfer from ferrous iron to hydrogen peroxide, generating a ferryl intermediate (FeO^{2+}) that typically releases a highly oxidizing hydroxyl radical.

Glycyl radical
a protein glycine residue from which a hydrogen atom has been removed, leaving an unpaired electron. It is used to initiate free-radical reaction mechanisms.

Hydrogen peroxide (H_2O_2)
an oxidant generated by acquisition of two electrons by molecular oxygen.

Hydroxyl radical (HO•)
a highly reactive radical generated by reduction of hydrogen peroxide.

Iron–sulfur clusters
enzyme cofactors most commonly consisting of either two or four matched iron and sulfur atoms; they catalyze electron-transfer or surface-chemistry reactions.

Reduction potential
the tendency of a molecule to receive electrons from other chemical species. A high (positive) potential signifies a high affinity for electrons.

Superoxide (O_2^-)
a radical species generated by acquisition of an electron by molecular oxygen.

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Highlights

Reactive oxygen species (ROS) attack iron-dependent enzymes that contemporary microbes inherited from their anoxic ancestors.

Microbes that adapted to live in oxic environments evolved layers of defenses against ROS, rather than discarding iron-centered metabolism.

Molecular oxygen directly damages the radical species and low-potential metal centers that are critical for optimal anaerobic metabolism, and these injuries may be more important than ROS stress in constraining anaerobes to anoxic environments.

Anaerobes periodically confront oxygen, and they have developed esoteric tactics to minimize injuries to some of their most vulnerable enzymes.

Outstanding Questions

What was the order in which microbes evolved defenses against oxidative stress?

How do bacteria dynamically respond to fluctuations in local oxygen levels?

Has evolution reached an end point, or are new oxidative defenses arising and being distributed today?

How do microbes exploit the oxidative vulnerabilities of their competitors?

Can bioengineering enable classic anaerobes to be employed in aerobic fermentations?

Box 1.

How Do ROS Damage Iron Enzymes?

The $[4Fe\text{-}4S]^{2+}$ cluster-dependent dehydratases are prominent targets of ROS [32–34] (Figure IA). These enzymes employ a solvent-exposed iron atom of their cluster to bind substrate, initiating a non-redox reaction in which the cationic iron atom stabilizes an oxyanionic reaction intermediate. During oxidative stress, superoxide binds the exposed iron atom and abstracts a single electron [32,33]. The reaction generates an unstable $[4Fe\text{-}4S]^{3+}$ species that quickly releases the key ferrous iron atom, rendering a $[3Fe\text{-}4S]^{+}$ species that is catalytically inactive. Dihydroxyacid dehydratase, aconitase, and fumarase are examples of such enzymes [33]. Hydrogen peroxide inactivates the same enzymes through a similar process [34]. Its oxidation of the exposed iron atom apparently forms a ferryl $[FeO]^{2+}$ species. When formed from loose iron, the ferryl species decays to a hydroxyl radical; however, when generated from a cluster the ferryl intermediate abstracts a second electron from the cluster itself. A ferric iron atom is released, and again a $[3Fe\text{-}4S]^{+}$ cluster results. The rate constants of cluster oxidation by superoxide and hydrogen peroxide are approximately 10^6 M^{-1} s^{-1} and 10^4 M^{-1} s^{-1}, respectively [33,34,56].

Wild-type cells use scavenging enzymes to minimize the intracellular level of ROS, but calculations suggest that, in aerobic *E. coli*, these enzymes are nevertheless oxidized every 30–60 min. Because the oxidation events do not damage the polypeptide, cells can repair the clusters by providing iron and electrons, with a half-time of ~5 min [89]. However, the clusters of some enzymes can degrade beyond the $[3Fe\text{-}4S]^{+}$ state, and the cluster must be rebuilt from scratch [90,91]. Iron–sulfur clusters are normally built upon the IscU protein scaffold by the concerted actions of the Isc cluster-assembly system, and they are then transferred to client proteins. During exposure to H_2O_2, *E. coli* induces the alternative Suf cluster-assembly complex, which, unlike the housekeeping Isc machinery, can assemble new clusters in an oxidizing environment [92,93]. Other defensive tactics include the induction of metal-free dehydratase isozymes, albeit with a loss of enzyme efficiency [94–96]. Some organisms have evidently adjusted to oxidizing habitats by evolving dehydratases whose active sites use $[2Fe\text{-}2S]^{2+}$ rather than $[4Fe\text{-}4S]^{2+}$ clusters [97]. The smaller clusters feature iron atoms that are formally in their ferric states and therefore resist further oxidation [33].

ROS also attack mononuclear iron enzymes that use single Fe(II) atoms (Figure IB). Such enzymes include some epimerases, dehydrogenases, deformylases, and deaminases. Superoxide and hydrogen peroxide both oxidize the atom to its ferric form, causing its release [30,31]. The reaction with superoxide produces a simple apoprotein that cells can directly remetallate. If the process recurs continuously, however, the protein may inadvertently bind a competing metal, such as zinc, which confers much less activity. The reaction of Fe(II) enzymes with hydrogen peroxide similarly generates an apoprotein – but it also results in the formation of hydroxyl radical (HO•). This highly reactive species is released into the active site where it can oxidize the polypeptide and irreversibly inactivate the enzyme. With *E. coli* ribulose-5-phosphate 3-epimerase, about 20% of iron-oxidation events lead to covalent damage – and during protracted stress, the entire

enzyme population is eventually inactivated. Some enzymes, however, employ a cysteine residue as an iron ligand; the cysteine quenches the $[FeO]^{2+}$ before it releases the hydroxyl radical and thereby protects the polypeptide.

Some bacteria withstand hydrogen peroxide stress by importing manganese to replace iron in mononuclear enzymes [54]. Manganese is catalytically less effective than iron, but it does not react with hydrogen peroxide and therefore allows enzyme activity to persist even in oxidizing environments.

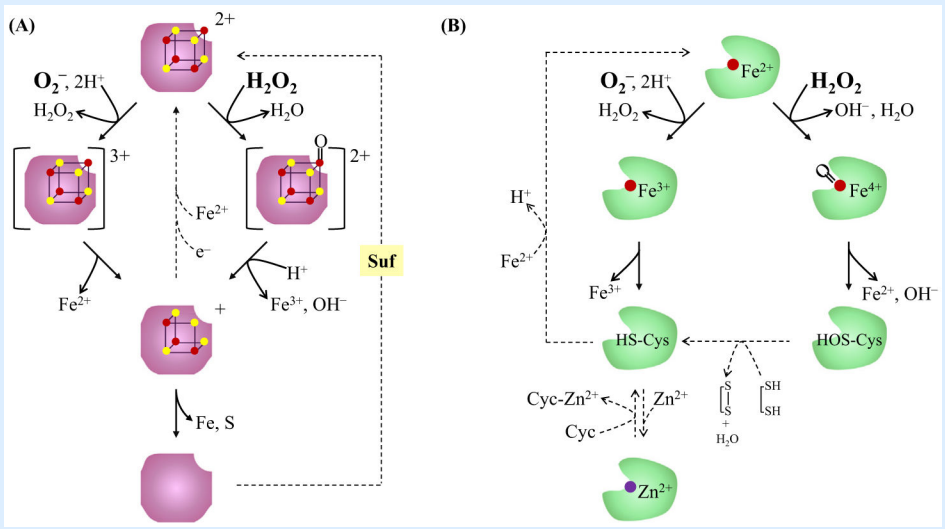

Figure I.

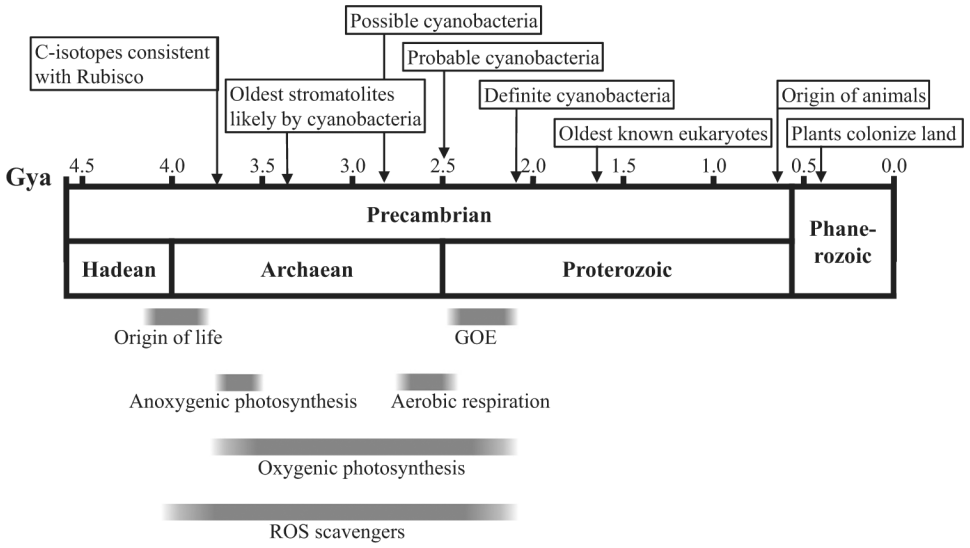

Figure 1. The Timetable of Evolution of Major Cellular Features.

The data are inferred from fossils, environmental proxies, and high-resolution geochronology [16,98,99]. Bottom. Life evolved for 1.5 billion years (Gya) prior to the Great Oxygenation Event (GOE). Both reactive oxygen species (ROS) scavenging and aerobic respiration may have pre-dated the GOE, suggesting that significant amounts of oxygen may have accrued locally prior to its general accumulation in the atmosphere. Abbreviation: Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

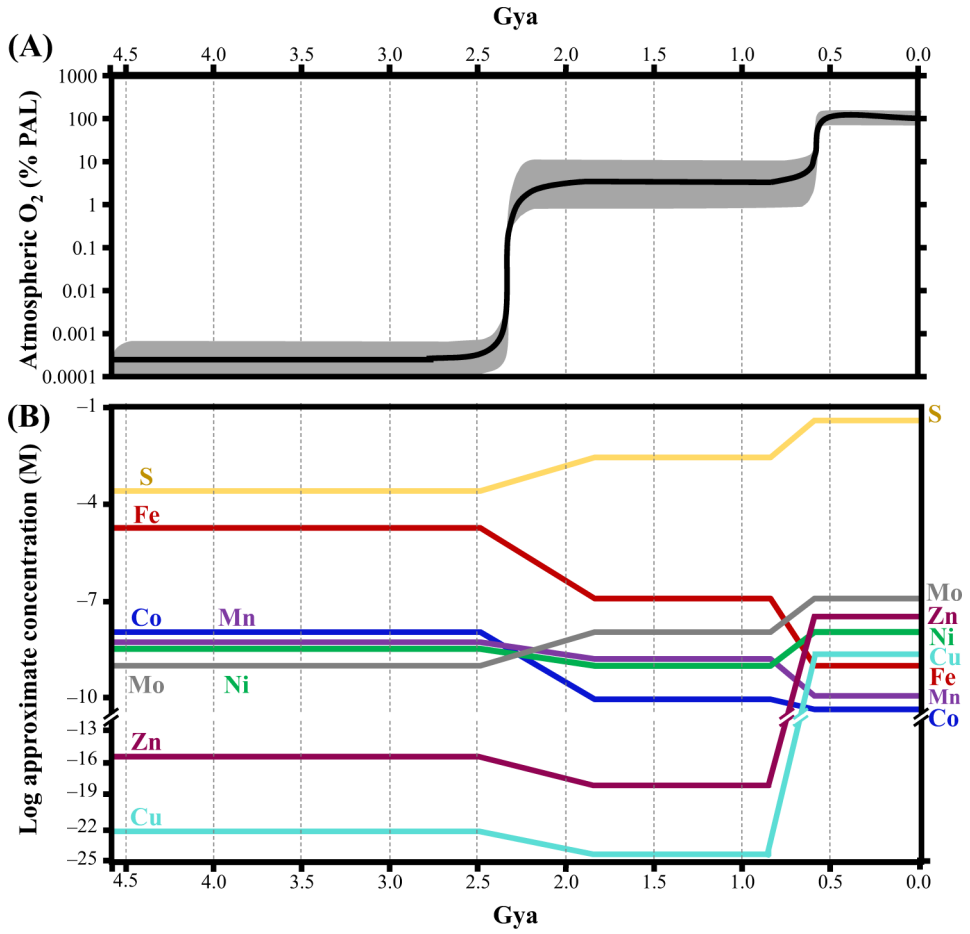

Figure 2. Changes in Atmospheric Oxygen and Oceanic Elemental Concentrations over Time.

(A) The prevailing view of the concentration of atmospheric oxygen suggests three steady states that roughly correspond to Archaean, Proterozoic, and Phanerozic eras. The gray areas show the range provided by geological proxies [13]. (B) The approximate concentrations of elements in the ocean are inferred from geochemical models and sediments, as summarized by Anbar [2]. A different pattern for changes in Ni and Zn concentrations was recently suggested by Robbins *et al.* [100]. Note the orders-of-magnitude decline in iron and the rise in copper. Abbreviations: Gya, billion years ago; PAL, present atmospheric level.

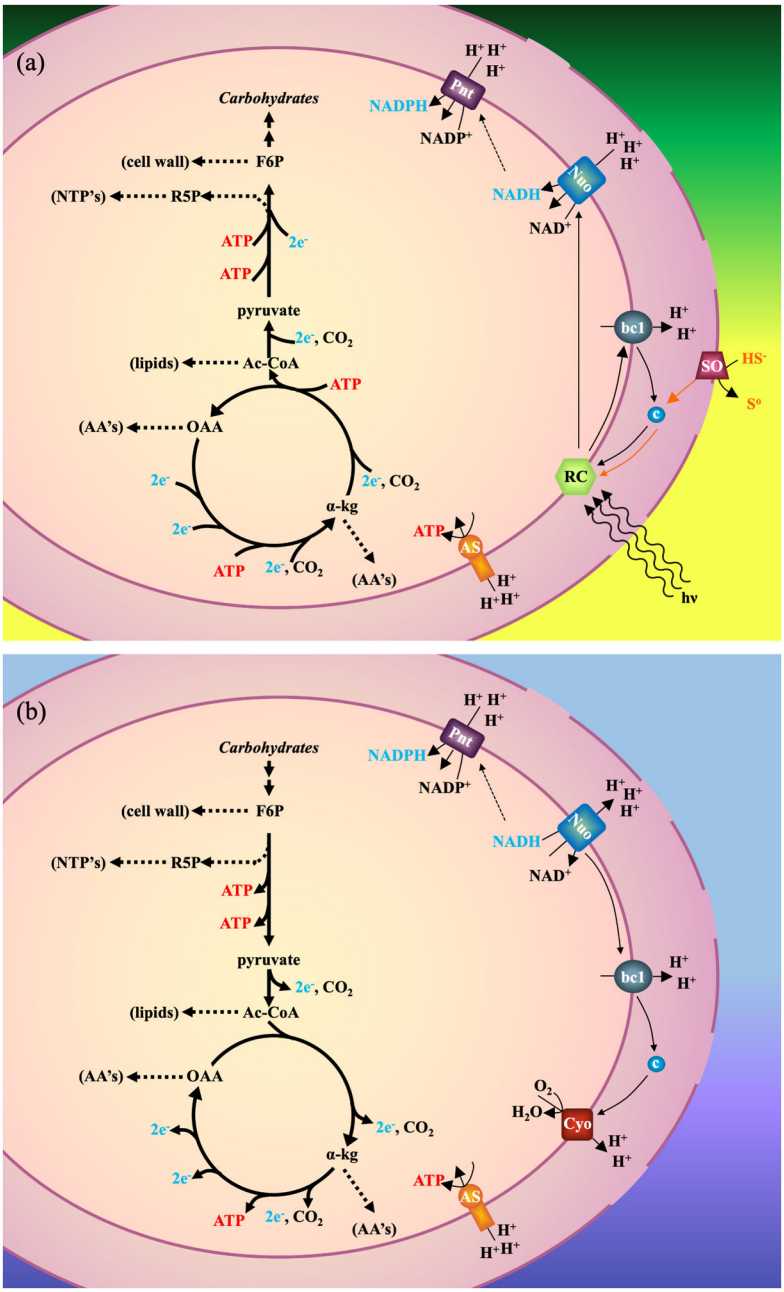

Figure 3. Few Steps Were Required to Evolve Aerobic Oxidizers.

(A) The green-sulfur photosynthetic bacterium *Chlorobium* lives in hypoxic, sulfur-rich waters under solar radiation – an environment that may resemble that in which similar bacteria lived on the ancient Earth. Its photocycle generates a protonmotive force that is used to both generate ATP and push electrons uphill through the bc1 and NADH dehydrogenase complexes, ultimately producing NADH as a cellular reductant. Carbon dioxide is assimilated by a reverse tricarboxylic acid (TCA) cycle, and larger biosynthetic precursors are produced by gluconeogenesis. The role of the TCA cycle and gluconeogenesis is to enable biosynthesis. (B) To evolve bacteria that oxidize carbohydrates, mass action need only reverse glycolysis, the TCA cycle, and the respiratory chain, while a single new

enzyme – cytochrome oxidase – delivered the electrons to oxygen. Thus, most components of aerobic respiratory chains pre-dated the oxic world. Abbreviations: α-kg, α-ketoglutarate; AAs, amino acids; Ac-CoA, acetyl coenzyme A; AS, ATP synthase; bc1, ubiquinol cytochrome *c* oxidoreductase; *bo* oxidase; c, cytochrome *c*; Cyo, cytochrome F6P, fructose-6-phosphate; NTPs, nucleoside triphosphates; Nuo, NADH dehydrogenase 1; OAA, oxaloacetate; Pnt, transhydrogenase; R5P, ribulose-5-phosphate; RC, reaction center; SO, sulfide oxidase.

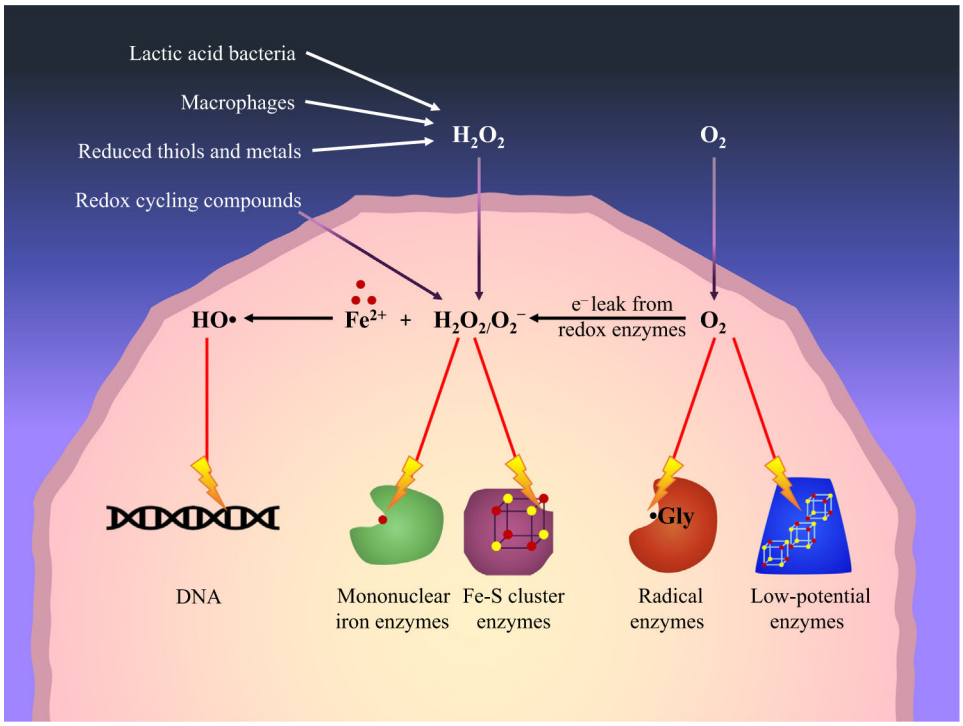

Figure 4. An Overview of Damage Caused by Molecular Oxygen and Its Reactive Species, Hydrogen Peroxide and Superoxide.

Oxygen freely equilibrates across cell membranes. Exogenous sources (lactic acid bacteria, macrophages, reduced thiols and metals) and endogenous sources (electron leak from redox enzymes and redox-cycling compounds) generate reactive oxygen species (ROS). The Fenton reaction between hydrogen peroxide and loosely bound intracellular iron forms hydroxyl radicals that can damage DNA. Hydrogen peroxide and superoxide disrupt enzymes that employ solvent-exposed Fe(II) or [4Fe-4S] cofactors. Molecular oxygen directly inactivates radical-based and low-potential enzymes.