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The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium

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Abstract

Oxic environments are hazardous. Molecular oxygen adventitiously abstracts electrons from many redox enzymes, continuously forming intracellular superoxide and hydrogen peroxide. These species can destroy the activities of metalloenzymes and the integrity of DNA, which forces organisms to protect themselves with scavenging enzymes and repair systems. Nevertheless, elevated levels of oxidants quickly poison bacteria, and both microbial competitors and hostile eukaryotic hosts exploit this vulnerability by assaulting them with peroxides or superoxide-forming antibiotics. In response, bacteria activate elegant adaptive strategies. In this Review, I summarize our current knowledge of oxidative stress in *Escherichia coli*, the model organism for which our understanding of damage and defence is most well-developed.

Microbial life first evolved in a world devoid of oxygen and rich with reduced iron[1]. By three billion years ago the extant microbial life forms shared basic biochemical mechanisms and a common metabolic plan, which persist today. The subsequent oxygenation of the atmosphere by photosynthetic organisms created a crisis: oxygen is a reactive chemical, and organisms had to devise strategies to defend themselves against it.

Molecular oxygen is small and non-polar, and it diffuses across typical biological membranes as quickly as through water[2]. Consequently, even the most active cells cannot respire quickly enough to lower the intracellular oxygen concentration substantially below the concentration immediately outside the cell. Some microorganisms escape oxidative stress by residing in anaerobic microhabitats; all others must contend with intracellular molecular oxygen. Cursory examination shows that the ability to do so varies widely: obligate anaerobes cannot tolerate oxygen at all, microaerophiles require that oxygen stays within a low-micromolar concentration range and aerobes thrive in air-saturated fluids. However, virtually all of these microorganisms suffer poor growth, elevated mutagenesis or even death when they are exposed to oxygen levels that exceed those of their native habitats. This also holds true for the facultative anaerobe *Escehrichia coli*[3].

The nature of the underlying injuries is not self-evident, since amino acids, carbohydrates, lipids, and nucleic acids—the structural molecules from which organisms are made—are essentially unreactive with oxygen. In the 1950s, Gerschman *et al.* suggested that oxygen toxicity might derive from the same events that underlie the toxicity of ionizing radiation: the formation of partially reduced oxygen species (ROS)[4]. The four-electron reduction series of molecular oxygen is depicted in Fig. 1A and shows that the addition of consecutive

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electrons generates superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO·). The lethal effects of ionizing radiation had recently been shown to derive from hydroxyl radicals, and so Gerschman's idea seemed plausible. It was not immediately obvious how intracellular oxygen might obtain the three electrons that could reduce it to HO, but catalases and peroxidases (enzymes that degrade H_2O_2) had long been recognized to be ubiquitous among aerobic organisms[5]. The existence of these enzymes implied that organisms must somehow routinely encounter H_2O_2 and that, were it not scavenged, H_2O_2 would harm the cell. In 1969 McCord and Fridovich reported the existence of an enzyme that dismuted superoxide (O_2^-), and so a similar inference was drawn for this molecule[6].

These ideas were finally confirmed when mutants of *E. coli* were generated[7]. Strains that lack cytoplasmic superoxide dismutase performed as well as their wild-type parents in anaerobic cultures, but they grew poorly in aerobic media. Analogous results were later observed for strains that lacked the primary catalases and peroxidases[8]. These studies set the stage for three fundamental questions that continue to frame the field: First, how do O_2^- and H_2O_2 arise inside aerobic cells? Second, what types of damage do they create? And finally, what strategies do cells use to defend themselves against them? In this Review, I discuss the endogenous and exogenous sources of H_2O_2 and O_2^- and describe the damage that they can produce in the *E. coli* cell. Furthermore, the defence strategies and repair programmes that *E. coli* has evolved to survive exposure to these toxic species are delineated.

Sources of intracellular O_2^- and H_2O_2

Molecular oxygen has an even number of electrons, but the final two reside in discrete orbitals as unpaired, spin-aligned electrons. This arrangement constrains oxygen to accept electrons one at a time[9]. Because its univalent reduction potential is slightly negative (−0.16 V; Fig. 1A), its affinity for that first electron is low, and so oxygen can take electrons only from good univalent electron donors, such as metal centers, flavins, and quinones. Such cofactors are prominent electron carriers in respiratory chains, and indeed both O_2^- and H_2O_2 have been detected as trace products when submitochondrial particles or inverted bacterial membranes respire *in vitro*[10, 11]. The flavins of dehydrogenases were subsequently identified as the primary sources of the O_2^- and H_2O_2[12, 13]. However, the rate at which cells produced H_2O_2 was not substantially diminished for mutants lacking respiratory enzymes, which led to the conclusion that both O_2^- and H_2O_2 are primarily produced by the accidental autoxidation of non-respiratory flavoproteins[14, 15]. Indeed, such flavoproteins are found throughout metabolism, and a wide variety of them release ROS *in vitro*, including glutathione reductase, lipoamide dehydrogenase and glutamate synthase[16–18].

Flavoprotein autoxidation occurs when molecular oxygen adventitiously collides with the dihydroflavin of the reduced enzyme (Fig. 1B). Resultant electron transfer generates O_2^- and a flavosemiquinone species. Sometimes the O_2^- immediately diffuses away, but most of the time a second electron transfer occurs before O_2^- escapes the active site, and H_2O_2 is the species that enters the bulk solution. Thus such enzymes are probably responsible for both O_2^- and H_2O_2 production. The autoxidation rates of flavoenzymes vary, since they depend upon the degree of flavin exposure, the flavin midpoint potential, and the residence time of electrons on it[19]. Therefore, it is plausible that the degree of ROS stress that an organism

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experiences depends on the titres of the most autoxidizable enzymes in the cell. For example, fumarate reductase is an abundant anaerobic respiratory enzyme that reacts unusually rapidly with oxygen, and it is the predominant source of O_2^- when facultative and obligately anaerobic bacteria enter aerobic habitats[15, 20]. Because the rate of enzyme autoxidation also depends on collision frequency, the pace of ROS formation is greater when oxygen concentration is high[14]—a fact that explains the toxicity of hyperoxia. In *E. coli* a minor fraction of ROS formation derives from the autoxidation of menaquinone, the low-potential electron carrier in the respiratory chain[21]. Like redox enzymes, it autoxidizes in proportion to the oxygen concentration.

Only 0.1–1% of the electron flux through any particular enzyme is likely to be intercepted by oxygen. However, measurements of H_2O_2 efflux from scavenger-deficient cells indicate that the aggregate rate of H_2O_2 formation inside aerobic *E. coli* is 10–15 μM/s[14]. Superoxide formation is estimated to be about 5 μM/s[24]. Because O_2^- and H_2O_2 react very rapidly with vulnerable targets (below), these rates are high enough to require the synthesis of ample scavenger enzymes (Box 1).

Scavenging enzymes

E. coli contains three superoxide dismutases (SODs): cytoplasmic iron- and manganese-cofactored enzymes (FeSOD and MnSOD) and a periplasmic copper-zinc-cofactored enzyme (CuZnSOD). Superoxide cannot cross membranes, so scavenging enzymes must be located within the cellular compartment that they are intended to protect. Periplasmic O_2^- formation has been detected during exponential growth[21], but apparently it is not harmful enough to warrant periplasmic SOD synthesis, as CuZnSOD is only synthesized when cells enter stationary phase[22]. To date no phenotype has been reported for *E. coli* mutants that lack it[23].

The cytoplasmic SODs are abundant enough to keep steady-state O_2^- at a sub-nanomolar concentration (Box 1)[24]. Titration studies have shown that this high SOD activity is necessary to avoid enzyme damage and to ensure vigorous growth[25]. The two isozymes (FeSOD and MnSOD) are coordinately regulated in response to iron levels. The iron-loaded Fur repressor blocks MnSOD synthesis when iron levels are high; when iron levels are low, the deactivation of Fur stimulates both MnSOD synthesis and transcription of the RyhB sRNA, which triggers degradation of the FeSOD message[26, 27]. Control by Fur also ensures that the MntH manganese importer is induced, thereby enabling activation of MnSOD[28]. Together, this arrangement ensures that FeSOD is the default isozyme and that MnSOD is synthesized when FeSOD cannot be activated. MnSOD synthesis is also stimulated whenever superoxide-generating antibiotics are present, with the control being exerted by the SoxRS system (described below)[29, 30]. SoxRS is not active during normal aerobiosis, in the absence of these exogenous O_2^- sources.

The picture of H_2O_2 scavenging is more complex. Over the years, researchers have identified a handful of *E. coli* enzymes that can degrade H_2O_2 *in vitro*. Of these only three have important roles *in vivo* under standard culture conditions: alkyl hydroperoxide reductase (Ahp), catalase G and catalase E[8]. Ahp is a two-component thiol-based peroxidase

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that transfers electrons from NADH to H_2O_2, thereby reducing it to water. It is the primary scavenging enzyme under routine growth conditions, as evidenced by the fact that in *ahpCF* null mutants enough H_2O_2 accumulates to activate the OxyR H_2O_2-stress response. Catalase G, which is encoded by *katG*, belongs to the catalase/peroxidase family and is only weakly expressed in exponential cells. However, OxyR strongly induces both *ahpCF* and *katG* when cells are stressed by exogenous H_2O_2[31]. Catalase E is strongly expressed only in stationary-phase cells, as it is induced by the RpoS system[32].

Why are so many enzymes used to scavenge H_2O_2? Catalases are generally more problematic than peroxidases, since when H_2O_2 concentrations are low, their two-step catalytic cycle can stall with the heme in its intermediate ferryl/radical form. This ferryl/radical species is a potent oxidant, and if left unresolved it can abstract electrons from the surrounding polypeptide[33]. In fact, to minimize this problem, KatE-type catalases bind NAD(P)H as a rescuing reductant, while KatG-type catalases feature a channel that enables a variety of metabolites to approach and reduce the ferryl/radical form[34]. By contrast, Ahp does not form a dangerous oxidizing species, and so it is the more efficacious scavenger during low-level H_2O_2 stress. However, Ahp can degrade H_2O_2 only as quickly as metabolism provides NADH as its stoichiometric reductant, and so Ahp becomes saturated when intracellular H_2O_2 exceeds 20 micromolar or when catabolic substrates are scarce. Under these conditions, catalases, which do not require stoichiometric reductants, can turn over much more quickly than Ahp. Taken together, these constraints have prompted organisms to rely on Ahp when H_2O_2 levels are low, and on catalases when they are high or when cells are starved.

Many oxygen-tolerant bacteria have additional enzymes that exhibit peroxidase activity *in vitro*. *E. coli* contains thiol peroxidase[35], bacterioferritin comigratory protein (Bcp)[36] and a glutathione peroxidase homologue[37]. However, although these three enzymes are synthesized by *E. coli* under laboratory conditions, they seem to lack H_2O_2-scavenging ability *in vivo*, since *ahp katG katE* mutants degrade little H_2O_2[8]. On the basis of these observations, it is possible that the true physiological activities of these enzymes have not yet been correctly identified.

Exogenous oxidative stress and the OxyR and SoxR regulons

The basal scavenging systems described above are just barely sufficient to protect *E*. coli from endogenous O_2^- and H_2O_2 (Box 1). However, environmental circumstances can elevate the rates at which these oxidants enter or are formed in the cell, and under such conditions these basal defences become inadequate.

The OxyR system

Environmental H_2O_2 arises from various sources: the chemical oxidation of sulfur and reduced metals at anoxic-oxic interfaces; the photochemical formation of oxidants by flavins and other chromophores; the excretion of H_2O_2 by lactic acid bacteria that contain lactate and pyruvate oxidases; and the deliberate action of the antimicrobial NADPH oxidases of macrophages, amoebae and plants. Because H_2O_2 is small and uncharged, it crosses membranes at a moderate efficiency that is similar to that of water. In fact, whenever the

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extracellular H_2O_2 concentration exceeds 0.2 micromolar, the rate of influx into *E. coli* exceeds the rate of endogenous H_2O_2 formation[38]. Therefore, when bacteria enter H_2O_2-containing environments, oxidative stress is likely to occur.

This threat is sensed by OxyR, a transcription factor whose active-site cysteine residue reacts rapidly with H_2O_2[39, 40](Fig. 2). OxyR is normally inactive during routine aerobiosis, when intracellular H_2O_2 is ~ 50 nM. However, an intracellular concentration of ~200 nM is sufficient to drive OxyR into a disulfide-bonded form that actively promotes the transcription of a dozen operons around the chromosome. Because basal Ahp activity can establish a five-fold outside-to-inside gradient, 1 μM extracellular H_2O_2 suffices to activate the regulon[38]. Members of the regulon have been identified[41] (Fig. 2). OxyR induces the synthesis of catalase G and Ahp more than 10-fold in an effort to drive the H_2O_2 concentration back down to innocuous levels. Other members of the regulon are best understood in the context of the damage that H_2O_2 creates, and so their roles in H_2O_2 resistance are discussed below. Interestingly, an alternative mechanism of sensing and responding to H_2O_2 exists in many Gram-positive bacteria. In these organisms the PerR repressor is inactivated when H_2O_2 oxidizes its prosthetic iron atom[42]. Strikingly, this induces homologues of many of the same enzymes that OxyR controls. It is currently unclear why two distinct mechanisms of H_2O_2 sensing have arisen.

The SoxRS system

Superoxide differs from H_2O_2 in that it is a charged species at physiological pH (pK_A = 4.8), and so it cannot penetrate membranes[43, 44]. This means that cytoplasmic O_2^- must be formed inside the cell. However, hostile plants and bacteria manage to induce the production of O_2^- in the cytoplasm of other bacteria by excreting redox-cycling organic compounds—typically phenazines or quinones—which can passively enter the interior of the target cell[45, 46]. Once there, these compounds abstract electrons from low-potential metal centers, respiratory quinones and flavins. These compounds can then transfer the electrons to oxygen, thereby generating O_2^-. In phenazine- and plumbagin-treated *E. coli*, the rate of O_2^- formation can increase by ~100-fold[47].

Early investigators discovered that under these conditions MnSOD titres increase by more than ten-fold[46]. This response was subsequently shown to be driven by the SoxRS system, which is inactive during regular growth but becomes highly active when cells are exposed to a wide variety of redox-cycling compounds[29, 30]. SoxR is a homodimeric regulatory protein containing one sensory [2Fe-2S] cluster per subunit. During redox-drug exposure the cluster becomes oxidized. Both reduced and oxidized SoxR bind upstream of the *soxS* gene, but only the oxidized form stimulates transcription (Fig. 2). The SoxS protein then acts as a secondary transcription factor, enhancing the expression of many genes scattered around the chromosome, including *sodA*. [Please see REF. 48 for a full list.] When the inducing compounds are removed, the SoxR protein returns to its reduced state, largely due to electron transfer from the Rsx complex[49]. The extant SoxS is quickly degraded because its N-terminal tail is targeted by the Clp protease system, leading to an end to the response[50].

Initially, it was expected that O_2^- would be the oxidant that activates SoxR, but *in vivo* experiments strongly indicate that the redox-cycling compounds themselves are the primary

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physiological activators[51–54] and that O_2^- is a relatively ineffective activator. There is, however, disagreement in the literature over whether O_2^- can oxidize SoxR *in vitro*.[52, 55] One rationale for this arrangement is that redox-cycling compounds are toxic to cells even under anoxic conditions (where O_2^- cannot be made) because of their ability to oxidatively destabilize enzymes and to act as Michael acceptors. Thus, SoxRS needs to sense the threat even when O_2^- is absent.

Many of the SoxRS-induced proteins work to exclude redox-cycling compounds from the cytoplasm: by slowing their entry through modifications of the charge and porin content of the cell envelope (via the WaaZY proteins and MicF sRNA), by actively pumping them back out of the cell (AcrAB and TolC) or by chemically modifying them (NfsA and YgfZ)[56–61]. A few induced enzymes—glucose-6-phosphate dehydrogenase and NADPH:ferredoxin oxidoreductase—help replenish the NADPH pools that are depleted when the drugs oxidize NADPH-reduced enzymes[62]. Other components of the regulon serve to mitigate specific types of cell damage (discussed below). The aggregate SoxRS response has a strong impact on the ability of *E. coli* to tolerate redox-cycling compounds.

SoxRS-type systems exist in many bacteria, albeit sometimes in modified form. SoxR often activates regulon members directly, without the intermediacy of SoxS[63]. Furthermore, SoxR seems to control phenazine synthesis and export in many bacteria that excrete these compounds; in such organisms SoxR does not control SOD synthesis[51, 64, 65]. The benefits of phenazine excretion for the producing organisms are being actively investigated. In addition to the suppression of competitors, phenazines can solubilize iron[66], deliver electrons to insoluble oxidants[67] and act as signalling molecules[63]. The picture that emerges is that these redox compounds are widely used by plants and microorganisms and that they are widespread enough that many bacteria carry a SoxR-type system to defend themselves against them. Superoxide formation is one element of the stress that these compounds impose, which explains why targeted organisms include SOD induction as part of their response. It has also been suggested that traditional clinical antibiotics might trigger the endogenous formation of ROS and thereby contribute to bacterial death[68], but this idea has since been challenged by several studies[69–71].

Damage caused by O_2^- and H_2O_2

The idea that O_2^- might be toxic to cells was initially contentious, and in fact early studies failed to identify biomolecules that it could easily damage[72–75]. This problem was ultimately solved by investigations into the growth defects of superoxide-stressed cells. In 1976 Olen Brown reported that hyperbaric oxygen imposed several amino acid auxotrophies upon wild-type *E. coli*[3]. Ten years later Carlioz and Touati reported that SOD-deficient mutants exhibited similar phenotypes: they were unable to grow unless their medium was supplemented with branched-chain (Leu, Ile, Val), aromatic (Tyr, Trp, Phe) and sulphurous (Met, Cys) amino acids[7]. These observations implied that hyperoxia might exert its effects by accelerating O_2^- formation, and that O_2^- specifically disrupted these specific biosynthetic pathways. More recently, an analogous approach has revealed defects that arise when H_2O_2 accumulates in catalase/peroxidase-deficient cells[76].

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Inactivation of iron-sulphur-dependent dehydratases

The origin of the branched-chain auxotrophy of SOD-deficient *E. coli* was solved when Kuo and Fridovich showed that O_2^- destroys the catalytic [4Fe-4S] cluster of dihydroxyacid dehydratase (DHAD)[77]. This cluster serves to bind α,β-dihydroxyacid substrates and, acting as a Lewis acid, directly participates in the dehydration reaction (Fig. 3A). The problem is that O_2^- is electrostatically driven to bind the solvent-exposed cluster. Upon protonation O_2^- becomes a strong univalent oxidant (Fig. 1A), and it abstracts a single electron from the cluster (Fig. 3B)[78]. The oxidized cluster is unstable, and the substrate-coordinating iron atom dissociates from it, rendering the enzyme inactive. DHAD is just one member of a family of cluster-cofactored dehydratases, and other members of the family were subsequently found to be equally sensitive. These include aconitase and fumarases A and B of the TCA cycle, which explained the inability of SOD mutants to grow on TCA-cycle substrates like succinate and acetate[79–82].

Importantly, work in yeast demonstrated that SOD-deficient *Saccharomyces cerevisiae* suffer the same type of injuries. *S. cerevisiae sod1* deletion mutants lack cytoplasmic SOD and are consequently defective in the activities of two [4Fe-4S] dehydratases (isopropylmalate isomerase and homoaconitase), which leads to disrupted synthesis of branched-chain amino acids and lysine[83, 84]. Deletion of *sod2*, which eliminates mitochondrial SOD, also impairs aconitase activity and thereby growth on glycerol, which requires robust TCA-cycle function[85, 86]. These observations were gratifying, as they confirmed that the nature of oxidative damage in *E. coli* is likely to be predictive of the types of damage that oxidants induce in other organisms.

The rate constant for cluster damage by O_2^- (up to $5 \times 10^6\ M^{-1}\ s^{-1}$ [82]) is so high that even in wild-type cells, where abundant SOD keeps O_2^- at a miniscule 10^{-10} M concentration, the halftime for enzyme damage is ~20 minutes. This time frame is very short compared to the usual doubling time of cells in natural habitats, and so this situation is tolerable only because cells continuously repair the damaged clusters by reduction and re-metallation. It is not clear whether dedicated proteins assist the repair process. YggX—a member of the SoxRS regulon—can help[52, 87], although the mechanism by which it does so is unclear. A di-iron protein, YtfE, can assist repair when clusters are nitrosylated during nitric oxide stress, but it appears to be minimally expressed under other conditions[88].

Interestingly, two members of the SoxRS regulon encode superoxide-resistant dehydratases that can functionally replace the sensitive enzymes. Fumarase C, a non-iron-sulphur enzyme, is strongly induced when SoxRS detects redox-cycling compounds; its expression restores flux through the TCA cycle[25, 81]. Similarly, aconitase A—which has an iron-sulphur cluster but seems resistant to inactivation—replaces the highly sensitive housekeeping aconitase B[89, 90].

Hydrogen peroxide also attacks iron-sulphur dehydratases, although the chemistry differs (Fig. 3B). The ability of H_2O_2 to oxidize ferrous iron has long been recognized:

$$Fe^{2+} + H_2O_2 \rightarrow [FeO^{2+}] + H_2O \quad [FeO^{2+}] + H^+ \rightarrow Fe^{3+} + HO\cdot$$

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The overall reaction is dubbed the Fenton reaction[91]. The HO· radical is an extremely powerful oxidant (Fig. 1A) that reacts at nearly diffusion-limited rates with virtually all organic molecules. Similarly to O_2^-, H_2O_2 can directly ligand and oxidize the catalytic iron atom of dehydratase clusters, precipitating iron loss and enzyme inactivation. If a HO· radical were generated during this process, one might expect it to fatally damage the local active-site residues. Therefore, it was surprising to learn that when H_2O_2 inactivates these enzymes *in vitro*, it is possible to restore full activity by supplying iron and a reductant[92]. The reason is that before the ferryl radical (FeO^{2+}) can decay into a HO· radical, it abstracts a second electron from the cluster, converting the radical into an innocuous hydroxide anion (Fig. 3B). The end result is that although the enzyme is temporarily disabled by the release of ferric iron, a repairable $[3Fe\text{-}4S]^+$ species is left behind.

This damage mechanism pertains only to the small set of [4Fe-4S] enzymes that are dehydratases. In most iron-sulphur proteins, including those involved in electron-transfer reactions, the clusters are fully coordinated by polypeptide and are thereby protected from these oxidants, which must directly contact the cluster to oxidize it. However, experiments have shown that when cells are exposed to H_2O_2 for several generations, even non-dehydratase cluster enzymes qre at risk[93]. This is because H_2O_2 poisons the Isc system, which is responsible for the transfer of [4Fe-4S] clusters to newly synthesized apoenzymes[94]. The mechanism of poisoning is unclear, although it seems plausible that H_2O_2 attacks the nascent clusters on the IscU scaffold protein, which is required for the assembly of new clusters. In any case, *E. coli* compensates for this problem by using OxyR to induce the Suf system[95]. This alternative machinery also builds clusters, but somehow it assembles them and transfers them to client proteins without interference by H_2O_2.

Inactivation of mononuclear iron proteins

Superoxide and H_2O_2 also disable a family of enzymes that use a single iron atom as a prosthetic group[76, 96] (Fig. 4). As in dehydratases, these iron atoms directly bind substrate and, by stabilizing oxyanionic intermediates, catalyze a wide range of reactions. This family includes epimerases, dehydrogenases, deformylases and deaminases. Inactivation by H_2O_2 involves a Fenton reaction between H_2O_2 and the iron atom, with release of Fe^{3+}. The inactivation rate constants resemble those for the dehydratases, and as little as 0.5 μM intracellular H_2O_2 disables them. In the case of ribulose-5-phosphate 3-epimerase, after enzyme damage by a single bolus of H_2O_2, the oxidized enzyme can be largely reactivated by the addition of ferrous iron, but a minor fraction is irreversibly inactivated due to polypeptide damage by the HO· that is produced[96]. Cycles of repair and re-oxidation progressively cause the loss of all activity. Other enzymes, such as threonine dehydrogenase and peptide deformylase, use a cysteine residue as one of the iron-coordinating ligands. The ferryl radical apparently oxidizes this residue to a sulfenic acid, effectively quenching the HO· before it can be released. Sulfenates are easily reduced by cellular reductants and remetallation allows the damaged enzyme to be fully repaired.

Superoxide also oxidizes and releases the iron atoms of mononuclear enzymes (Gu and Imlay, uncited observations). Because this reaction does not form a strong oxidant such as ferryl or hydroxyl radicals, the polypeptide is wholly undamaged. However, inside cells

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repeated cycles of this process ultimately lead to mismetallation of the enzyme with zinc, an alternative metal that is reasonably abundant *in vivo*. Zinc is not as catalytically efficient as iron in these enzymes[76, 96], so there is a progressive decline in enzyme function.

E. coli has a clever strategy to maintain activity of these mononuclear enzymes during H_2O_2 stress: it replaces the iron atom with manganese[97]. The OxyR regulon triggers the strong induction of Dps (an iron-sequestering ferritin) and MntH (a manganese importer). Thus, when the cell is under oxidative stress, the intracellular level of manganese increases. When it is bound in the mononuclear site, manganese serves as a H_2O_2-resistant cofactor that is almost as catalytically active as iron itself.

DNA damage

In some circumstances the most consequential impact of oxidative stress is mutagenesis. Neither H_2O_2 nor O_2^- can damage DNA directly, but both SOD and catalase/peroxidase mutants exhibit high mutation rates[98, 99]. In the case of H_2O_2 the reason is obvious: by reacting with the cellular pool of unincorporated iron, some of which adventitiously associates with DNA[100], H_2O_2 produces hydroxyl radicals that can oxidize both base and ribose moieties of the DNA, giving rise to a wide variety of lesions[101, 102]. Guanine is disproportionately damaged because its lower reduction potential allows its electrons to hop to electron holes in nearby oxidized base radicals[103]. For example, although an adenine residue might be the initial site of hydroxyl-radical attack, immediate electron movement from a neighboring guanine “repairs” the adenine radical, and a lesion ultimately resides on the guanine. One common product is 8-hydroxyguanine, which is highly mutagenic owing to its ability to base pair with adenine in a way that eludes the intrinsic mispair detection system of DNA polymerases[104]. By contrast, thymine oxidation produces lesions that are more likely to be non-coding; such lesions block polymerase progression and tend to be lethal rather than mutagenic[105]. Similarly, the oxidation of ribose moieties generates polymerase-blocking single-strand breaks with 3’ glycolate residues 5’ to the break. These residues cannot be used as primers and must be removed prior to repair (see below).

Because iron is the co-reactant in the Fenton reaction, the rate of DNA damage is elevated when iron levels are high. For this reason superoxide-stressed cells, such as *fur* mutants that oversynthesize iron importers, exhibit high levels of DNA damage[106, 107]. Conversely, during periods of H_2O_2 stress OxyR induces Dps,[108–110] a ferritin-class protein which strongly suppresses the amount of DNA damage by sequestering the unincorporated iron[99]. YaaA, another OxyR-induced protein, also has a role in controlling iron levels, although the mechanism involved is unknown[111]. And interestingly, because Fur is itself a mononuclear iron protein, it tends to lose activity during both O_2^- and H_2O_2 stresses, which could potentially lead to derepression of iron-acquisition systems and the disastrous import of more iron[112]. To avoid this situation, both the SoxRS and OxyR systems stimulate the synthesis of even more Fur and thereby curb the synthesis of iron importers[113].

Repair of damaged DNA

When DNA damage occurs, repair is essential. FAPY glycosylase (also known as MutM and Fpg), endonuclease IV and endonuclease VIII initiate the excision of oxidized bases[114–116].

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These enzymes are not highly specific for particular lesions; instead, they scan for helical distortions, thereby enabling removal of the many disparate adducted bases that oxidation can produce. Exonuclease III and endonuclease IV excise fractured ribose moieties and restore a 3' primer for DNA-polymerase I-driven repair synthesis[105]. When excision systems fail to recognize lesions or when replication forks overtake adducts before they can be repaired, post-replication recombination is the back-up strategy. Strains that lack *rec* genes are hypersensitive to exogenous H_2O_2, and under aerobic conditions *recA* mutations are synthetically lethal with either catalase/peroxidase or SOD deficiency[99, 106]. In fact, strains that are deficient in both recombinational and excision repair strategies, such as *recA xth* and *polA recB* mutants, are fully viable only in anaerobic media. Thus, routine aerobiosis creates enough oxidative DNA lesions that repair is an essential function.

RecA protein also controls expression of the SOS system. This regulon includes the *sfiA* (*sulA*) gene, which encodes a protein that suspends cell septation until replication resumes and daughter chromosomes can be formed. Error-prone lesion by-pass is a final option to cope with DNA damage, allowing replication to proceed past lesions that have not been repaired. It is likely that this process is facilitated by one or more of the translesion polymerases[117]. Two of these—Pol IV and PolV, encoded by *dinB* and *umuCD*, respectively—are regulated by the SOS system. These genes are induced later than the other repair systems, presumably because mutagenesis is a collateral outcome that is appropriate only when excision and recombinational efforts have failed.

Interestingly, no DNA repair activity is controlled by OxyR, and only endonuclease IV (encoded by *nfo*) is regulated by SoxRS[30]. Why is this? DNA-repair processes are distinguished from other defensive functions in that DNA repair needs to continue after the oxidative stress itself has abated. Therefore, the SOS system continues to induce repair proteins as long as replication blocks persist. When *E. coli* is exposed to a high concentration of H_2O_2, the initial effect is a suspension of growth and metabolism due to enzyme inactivation. Only after the H_2O_2 is removed by extant peroxidase and catalase activities can metabolism resume—and it is at this point that the SOS system is activated, its member proteins are synthesized and DNA damage is addressed.

Oxidation of cysteine residues or lipids?

Biochemists long ago recognized that dissolved oxygen in cell extracts can promote the oxidation of protein cysteinyl and methionyl residues and the peroxidation of lipids. Consequently, at the outset of investigations into bacterial oxidative stress, the expectation was that similar injuries would lie at the heart of cell dysfunction. However, to date none of the phenotypes of SOD- or catalase/peroxidase-deficient cells have been linked to such events. In fact, most cysteine residues react very sluggishly with O_2^- or H_2O_2—certainly orders of magnitude more slowly than iron[118]. Indeed, experiments that have detected general protein disulfide formation inside H_2O_2-stressed *E. coli* cells have typically resorted to millimolar concentrations of H_2O_2[119, 120]. (The active-site thiols of OxyR and Ahp are exceptionally reactive, for reasons that are unclear, and their oxidation inside cells can be detected after the application of low-micromolar H_2O_2[39].) Methionine is even less reactive. Thus, most proteins are not targeted by physiological doses of these oxidants.

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Why then do glutaredoxin 1, thioredoxin 2 and DsbG, redoxins that excel in reducing disulfide bonds, belong to the OxyR regulon? Glutaredoxin helps deactivate OxyR by reducing its disulfide bond once H_2O_2 stress has passed[121], but this does not explain why OxyR induces the other proteins. One possibility is that enzymes with hypersensitive cysteine residues are out there, but workers have not yet chanced upon them. Second, bicarbonate accelerates H_2O_2-driven thiol oxidation[122, 123], raising the prospect that in CO_2-rich environments, disulfide formation might be relevant. A third possibility is that these redoxins help restore the coordinating cysteine ligand that can be oxidized by the ferryl radical in the active sites of mononuclear iron enzymes (Fig. 4). But the most intriguing answer might be that the OxyR protein moonlights as a sensor of other stresses that modify protein thiols more effectively than H_2O_2. Recent work[124] has shown that during anaerobic growth, some nitric oxide escapes nitrate and/or nitrite reductases and then nitrosylates protein thiols, including the sensory cysteine residue of OxyR. The induction of the OxyR regulon protects *E. coli* from this stress—perhaps by providing redoxins that reductively release the NO. Thus, it may be wrong to assume that every member of the OxyR regulon provides an important defence against H_2O_2

Lipid peroxidation is a universal outcome of oxidative stress in eukaryotic systems, but this seems less likely in most bacteria. In the standard model of lipid peroxidation, the propagation step of the chain reaction requires that lipids be polyunsaturated. Most bacterial lipids contain only saturated and monounsaturated fatty acids[125], which are not prone to peroxidation in model systems[126]. A few studies have reported evidence that would appear to be consistent with the peroxidation of bacterial membranes, but more-specific analyses must be done to verify these data[37, 127, 128]. A recent study suggested that *Borrelia burgdorferi* is the exception that proves the rule: this intracellular bacterial pathogen acquires fatty acids from its eukaryotic host and can incorporate polyunsaturated ones. Upon examination, *B. burgdorferi* exhibited vulnerability to lipid peroxidation, whereas an *E. coli* control strain did not[129].

On-line summary

Excess oxygen can disrupt the growth of most organisms, but the underlying mechanisms of damage have proven difficult to unravel. The model bacterium *Escherichia coli* represents the best understood organism in terms of oxidative stress and is the focus of this Review.

Superoxide and hydrogen peroxide are formed within cells when molecular oxygen adventitiously acquires electrons from the reduced cofactors of flavoproteins.

Both O_2^- and H_2O_2 can oxidize the exposed iron-sulphur clusters of a family of dehydratases. This event destabilizes the clusters, and their consequent disintegration eliminates enzyme activity.

O_2^- and H_2O_2 also inactivate a variety of non-redox enzymes that employ single ferrous iron atoms as catalytic cofactors.

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DNA is damaged when H_2O_2 reacts with the intracellular pool of unincorporated iron. The iron that is released from oxidized metalloproteins enlarges this pool and accelerates this process.

The OxyR transcription factor detects modest increments in intracellular H_2O_2. It activates several responses that help preserve the activities of iron-sulphur and mononuclear metalloenzymes.

The SoxRS system detects redox-active compounds that are released by plants and some bacteria. These compounds can generate toxic doses of O_2^- and the SoxRS system acts primarily to minimize the amounts of these compounds inside the cell.

Future studies should aim to determine whether the knowledge gained from studying oxidative stress in the facultative anaerobe *E. coli* is applicable to other organisms, such as strictly aerobic and microaerophilic bacteria.

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Glossary

Spin-aligned electrons	Electrons in separate orbitals with the same spin quantum number. Two electrons must have opposite spins to reside in the same orbital.
Reduction potential	The measure of the thermodynamic affinity of a compound for an electron.
Metal centres	Redox enzymes commonly use the transition metals iron, copper, manganese, molybdenum, nickel and selenium for electron-transfer reactions.
Flavins	Organic cofactors bound to redox enzymes in the form of FAD (flavin adenine dinucleotide) or FMN (flavin mononucleotide). These are commonly used to mediate electron exchange between divalent electron donors and univalent acceptors.
Respiratory quinones	The lipid-soluble organic molecules that carry electrons between membrane-bound redox enzymes.
Hyperoxia	Oxygen concentrations above that of air (22% oxygen).
Chromophores	Light-absorbing compounds.
Thiol-based peroxidases	Enzymes that use a redox-active cysteine residue to reduce H_2O_2 to water.

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RpoS system	The regulon that is governed by the RpoS sigma factor. RpoS is activated in stationary phase and under many stress conditions that suppress growth.
Michael acceptor	An α,β-unsaturated carbonyl compound that is vulnerable to addition reactions by nucleophiles.
Half-time	In an exponential decay process, the time needed for conversion of half of the reactant to product.
Lewis acid	A molecular moiety that can share an electron pair provided by a donor compound.
Isc system	A multiprotein complex that assembles iron-sulphur clusters on a scaffold protein and then transfers them to client proteins.
Suf system	Like the Isc system, a protein complex that assembles and transfers iron-sulphur clusters to recipient proteins. The Isc and Suf systems are constituted of different proteins and the activity of the Suf system is more resistant to chemical stress and iron deficiency.
SOS system	The global response to DNA damage exhibited by many bacteria.
Peroxidation	Lipid damage in which peroxyl groups are added to unsaturated bonds, thereby disrupting lipid packing in the membrane.
Redoxins	Proteins that use their cysteine residues to deliver electrons to oxidants. Thioredoxins and glutaredoxins reduce disulphide bonds in cellular proteins.
Oxidation	An increase in oxidation state, typically upon electron transfer to another molecule or atom.
Reduction	A decrease in oxidation state, typically upon acceptance of an electron from another molecule or atom.

Biography

Jim Imlay received his Ph.D. from the University of California at Berkeley, where he studied the mechanism of oxidative DNA damage under the guidance of Stuart Linn. He performed postdoctoral research on mechanisms of ROS formation with Irwin Fridovich at Duke. Since 1992 he has been on the faculty of the University of Illinois at Urbana-Champaign.

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What's next?

For the past forty years, studies in *E. coli* have pioneered our understanding of the oxidative-stress problem with which all organisms contend. The current overview of damage by O_2^- and H_2O_2 is presented in Fig. 5. We now have basic ideas of how ROS are formed, what they can damage, and how *E. coli* defends itself against them. It is clear that ROS vulnerability results from the use of iron as an enzymic cofactor. Iron is chemically versatile, facilitating both redox and surface chemistry; there is no mystery as to why ancient organisms which lived in an anaerobic, iron-rich environment recruited it to charge a wide diversity of enzymes. The problem is that contemporary organisms have inherited the same metabolic pathways and seek to use them in oxygen-rich habitats.

This picture is still incomplete, but as it becomes clearer, the next challenge will be to test the extent to which this paradigm applies to other microorganisms. There are reasons to think that important differences will arise. As we learn which types of redox enzymes are most predisposed to autoxidation, we can ask whether the possession of high titres of such enzymes is what constrains some microorganisms to microaerobic or anaerobic lifestyles. Additional targets of oxidants apparently remain to be found, since SODs are found in many Gram-negative periplasms and in iron-free *Borrelia burgdorferi*, both of which lack the classes of iron enzymes that superoxide is known to damage. And even the ROS targets that are well-established in *E. coli* may not pertain to strict aerobes; the iron-cofactored enzymes that are vulnerable to ROS in this facultative anaerobe might be routinely cofactored by other metals in aerobes, transforming what is an adaptive defence in *E. coli* into a constitutive one. Differences in environmental circumstances undoubtedly prompted different solutions to the oxidative threat, and the ongoing efforts to uncover this diversity comprise the next big step in this field.

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Box 1. Reactive oxygen species by the numbers: teetering on the brink

Do H_2O_2 or O_2^- produce any damage in healthy aerobic *E. coli*, or has the evolution of scavenging enzymes fully solved the problem? Direct measurements indicate that intracellular H_2O_2 is formed at a rate of 10 μM/s[14]. Given the titre (~ 5 μM)[130] and rate constant ($k_{cat}/K_M = 4 \times 10^7\ M^{-1}\ s^{-1}$;[131]) of AhpC, the primary scavenger, the steady-state H_2O_2 concentration must be ~ 50 nM. This seems extremely low. However, the rate constants for reactions between H_2O_2 and the dehydratases and mononuclear enzymes are typically in the range of 10^3–$10^4\ M^{-1}\ s^{-1}$ [76, 92], which means that the half-time for enzyme inactivation by H_2O_2 must be as short as 20 minutes. Since the half-time for repair of dehydratase clusters is about 5 minutes[132], at any moment during aerobic growth a significant minority of the enzyme population is inactive. Any additional H_2O_2 stress will exacerbate the situation. Indeed, the OxyR system is calibrated to be activated if intracellular H_2O_2 reaches ~200 nM and growth defects become evident when levels rise to 400 nM[38, 96].

The rate of endogenous O_2^- formation is probably slightly lower (~ 5 μM/s)[24]. The titre (20 μM) and rate constant ($10^9\ M^{-1}\ s^{-1}$) of SOD means that steady-state O_2^- levels are ~ 0.2 nM, or fewer than 1 molecule per cell! Yet it is necessary for O_2^- to be this scarce: the rate constants for inactivation of dehydratases and mononuclear enzymes by O_2^- exceed $10^6\ M^{-1}\ s^{-1}$ [82], so this steady-state level of O_2^- should also cause an inactivation half-time of as little as 20 minutes. Indeed, experiments confirm that a modest decrement in SOD titre elicits enzymatic and growth defects[25]. Thus, there is nothing excessive about the high titres of scavenging enzymes: the cell needs everything it's got. By the same token, any additional stress, via the influx of H_2O_2 from the environment or O_2^- formation by redox drugs, easily pushes the cell over the edge. This is why the OxyR and SoxRS systems are necessary.

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Box 2. Confused about "oxidative stress" and "antioxidants"?

The term "oxidative stress" is unfortunately broad. This review focuses on the damage created by O_2^-, H_2O_2 and the hydroxyl radical. These are the oxidants most likely to plague aerobic organisms, but other oxidizing species can cause injuries that are both important and chemically distinct. Molecular oxygen itself can directly inactivate specialized free-radical and low-potential enzymes that are required for some forms of anaerobic metabolism[133, 134]. For example, in *E. coli* oxygen rapidly poisons pyruvate:formate lyase and the anaerobic ribonucleotide reductase, and among other organisms it can directly inactivate enzymes such as pyruvate:ferredoxin oxidoreductase and nitrogenase, which use low-potential iron-sulphur clusters to conduct difficult chemistry. These mechanisms of toxicity restrict some bacteria and archaea to anaerobic habitats.

Other compounds create stresses that similarly fall within the broader notion of oxidative stress. Singlet oxygen is a potent divalent oxidant that can be formed by excited chromophores in photosynthetic microorganisms and plants[135]. It can covalently damage both enzymes and lipids; carotenoids, which quench singlet oxygen, are crucial for protection. Disulphide stress is often created in laboratory organisms by the addition of diamide, a synthetic electrophile that oxidizes thiols without the involvement of oxygen species[136]. While diamide was initially thought to mimic the action of H_2O_2, at this point there is uncertainty over whether physiological H_2O_2 oxidizes many thiols; natural sources of errant disulphide bonds await identification. Hypochlorous acid (bleach) is a potent oxidant that is generated by the myeloperoxidases of neutrophils. It reacts especially rapidly with sulphurous amino acids and it seems that *E. coli* possesses both a transcriptional regulator[137] and a chaperone[138] that respond to it. Finally, even the redox-cycling compounds that stimulate superoxide formation exert their own direct effects by abstracting electrons from enzymic flavins and iron-sulphur clusters[52]. In all of these cases the particular chemistry of the oxidant results in distinctive physiological effects, and cells deploy different strategies to defend themselves. Thus, it is important that the stresses induced by different compounds are not equated.

So what is an "antioxidant"? Exogenous "scavenging" agents have virtually no chance of substantially diminishing the intracellular level of either superoxide or hydrogen peroxide, because the cell is already loaded with high titres of very efficient enzymes that do the same thing. Similarly, it seems improbable that exogenous agents can have much impact on intracellular hydroxyl-radical lifetimes, since cells are already crowded with biomolecules that react with them at nearly diffusion-limited rates. The upshot is that actual "antioxidants" must work through other mechanisms. Manganese can protect cells by substituting for iron in mononuclear enzymes (see text) and possibly by quenching nascent ferryl radicals before hydroxyl radicals are released[139]. Cell-penetrating iron chelators, such as dipyridyl and o-phenanthroline, bind unincorporated intracellular iron; they thereby block its participation in Fenton chemistry and prevent oxidative DNA damage. Supplements of thiols (such as acetylcysteine and thiourea) and reductants (such as ascorbate) have been widely used in experiments to diagnose "oxidative stress", but it is much less clear how they might help, since these species are not especially effective at

scavenging reduced oxygen species. One possibility is that whopping doses of thiols might penetrate cells and chelate iron.

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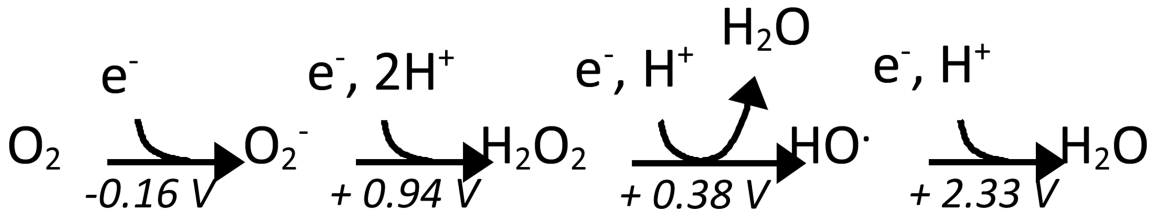
$$O_2^- + O_2^- + 2H^+ \xrightarrow{[SOD]} H_2O_2 + O_2$$

$$H_2O_2 + H_2O_2 \xrightarrow{[catalase]} O_2 + 2H_2O$$

$$NADH + H_2O_2 \xrightarrow{[Ahp]} 2H_2O$$

A.



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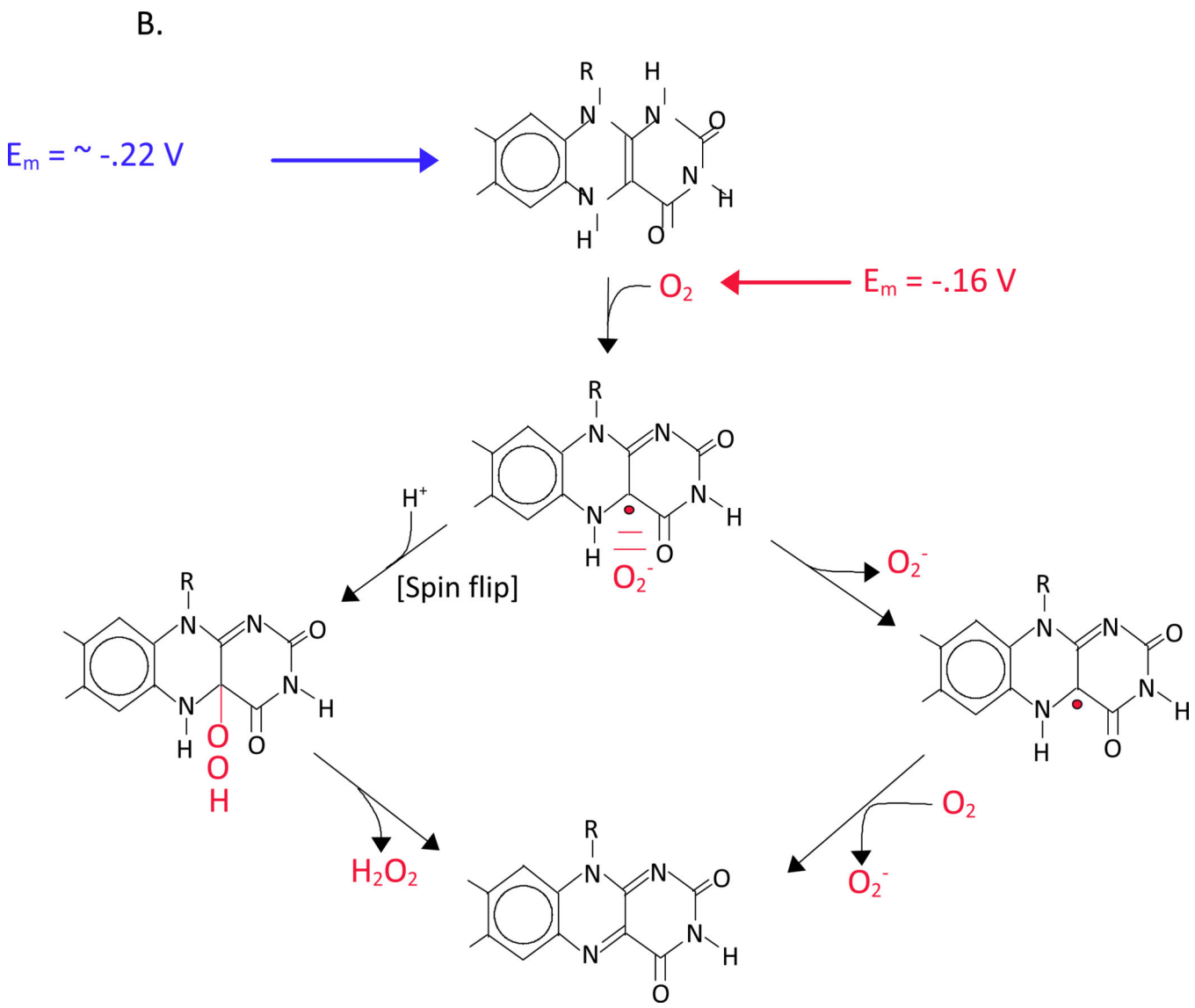

Fig. 1. The generation of O_2^- and H_2O_2

(a) The univalent reduction series of oxygen. The standard reduction potentials (pH 7) of molecular oxygen (O_2), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO·) indicate that the last three are potentially potent univalent oxidants, in contrast to O_2. The standard concentration of oxygen is regarded as 1 M. (b) Two pathways of adventitious dihydroflavin ($FADH_2$) oxidation on flavoproteins. Flavin autoxidation is possible because enzymic flavins commonly have univalent reduction potentials as low as that of molecular oxygen. The pathway to the left requires an electron spin flip by either the flavosemiquinone or superoxide, allowing adduction and ultimately H_2O_2 release[140]. The pathway on the right releases two consecutive molecules of superoxide to the bulk solution. The left pathway predominates in most enzymes studied to date.

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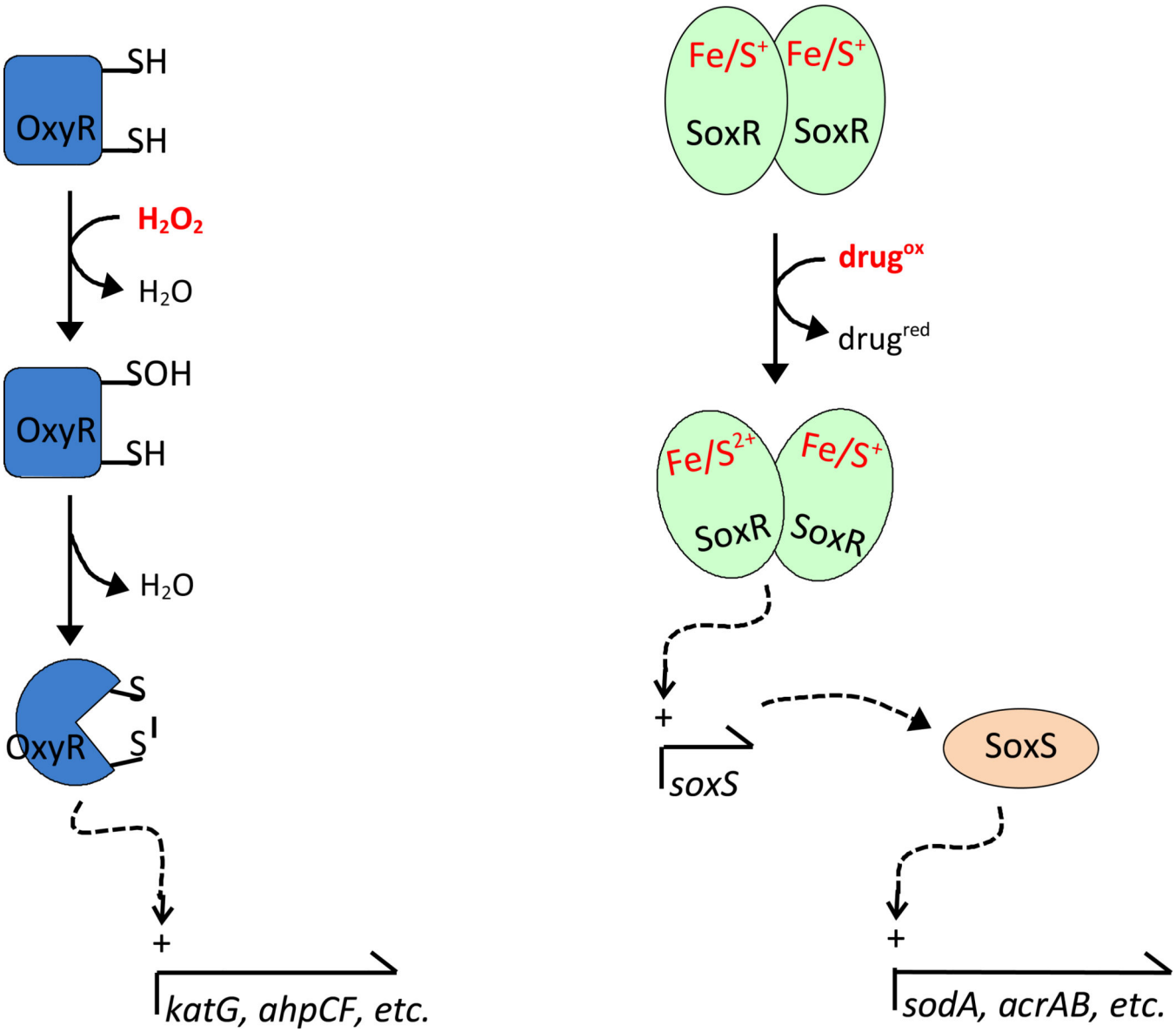

Fig. 2. Activation of redox-sensitive transcriptional regulators in *E. coli*

E. coli contains two defence systems that are induced under conditions of oxidative stress: the OxyR system (which responds to H_2O_2) and the SoxRS system (which responds to redox-active compounds). (a) Activation of the OxyR systems occurs when a sensory cysteine residue on the OxyR protein reacts rapidly with H_2O_2, forming a sulphenic acid moiety that then condenses with a resolving cysteine[40]. The resultant disulphide bond locks OxyR into a conformation that enables it to act as a positive transcription factor for regulon members, such as *katG* (encoding catalase G) and *ahpCF* (encoding Ahp), among other genes. (b) SoxR is a homodimeric transcription factor and each monomer contains an Fe-S cluster. The dimer becomes activated through the direct oxidation of these clusters by redox-active compounds[52], typically phenazines or quinones, which are produced by plants and bacterial competitors. Oxidized SoxR stimulates transcription of *soxS*, and the SoxS protein acts as a secondary transcription factor that goes on to activate expression of regulon

members, including *sodA* (encoding superoxide dismutase) and *acrAB* (encoding a multi-drug efflux pump), among a large array of other genes.

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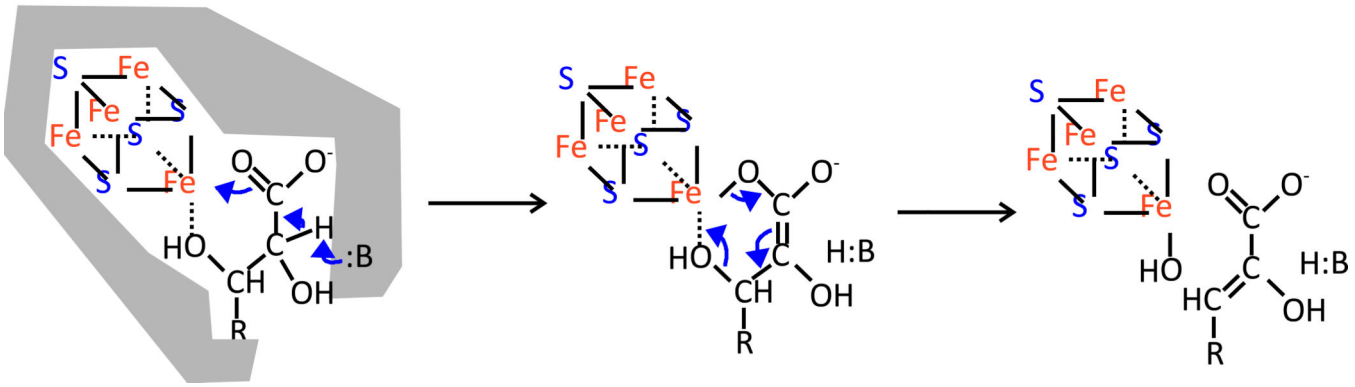

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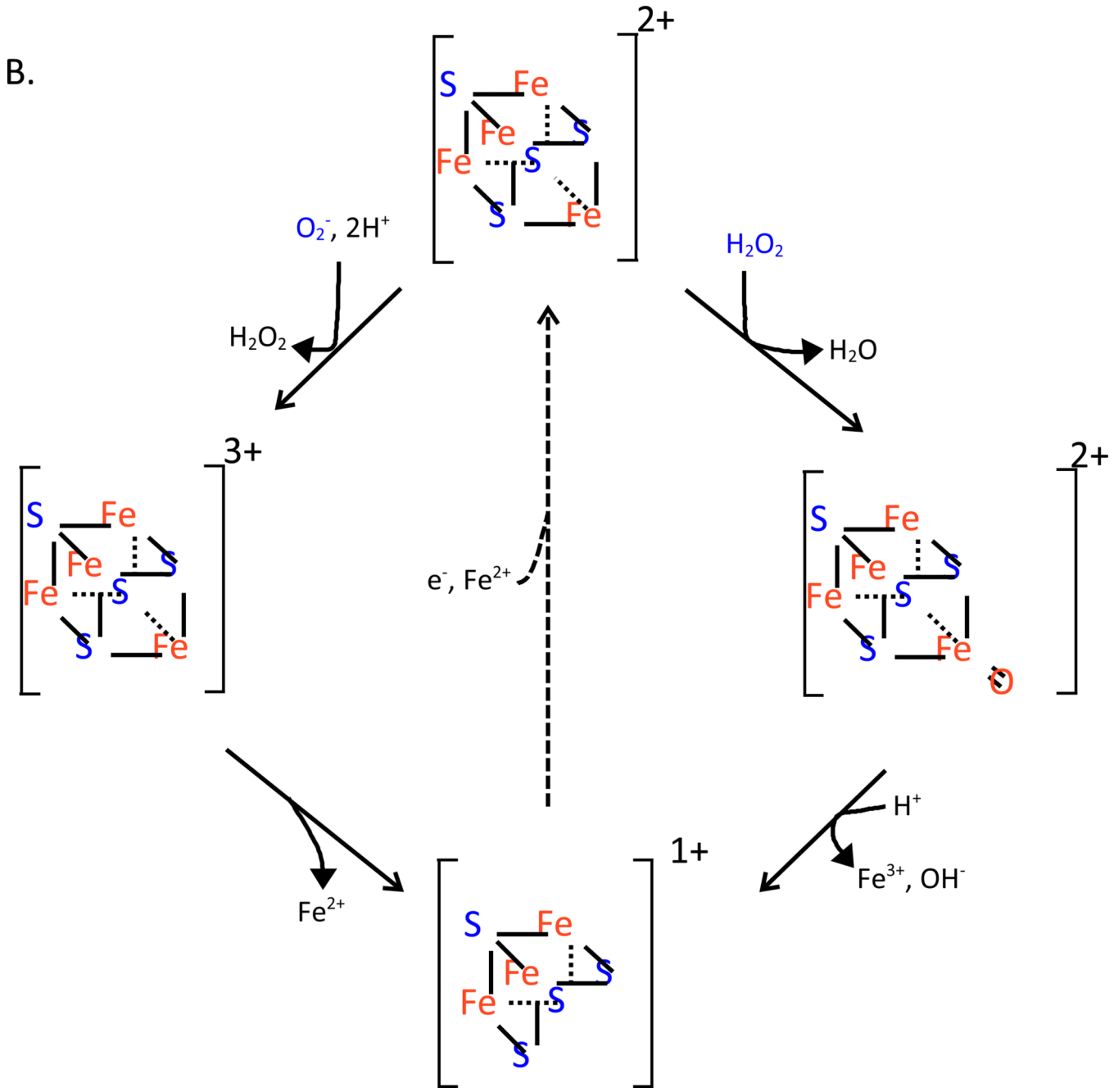

Fig. 3. The role and oxidative vulnerability of dehydratase [4Fe-4S] clusters

(a) Cellular dehydratases reversibly dehydrate α,β-dihydroxyacids, releasing enol products (shown) that subsequently tautomerize to α-ketoacid products (not shown). The cluster coordinates substrates through their β-hydroxyl and carboxylate groups. Deprotonation by a nearby base (B:) triggers hydroxide abstraction by the catalytic iron atom, comprising a net dehydration. Arrows denote electron shifts towards new bonding partners. (b) The left pathway shows the exposed cluster being oxidized by O_2^-, resulting in the formation of H_2O_2 and conversion of the cluster to an unstable $[4Fe\text{-}4S]^{3+}$ species that then releases ferrous iron (Fe^{2+})[82]. The loss of the catalytic iron atom eliminates enzyme activity. The

right pathway shows oxidation of the cluster by H_2O_2, which presumably creates a transient ferryl species that abstracts a second electron from the cluster. Ferric iron (Fe^{3+}) dissociates[92]. After damage by either oxidant, the resultant $[3Fe\text{-}4S]^+$ cluster can be reactivated *in vitro* and *in vivo* by reduction and remetallation (dashed line).

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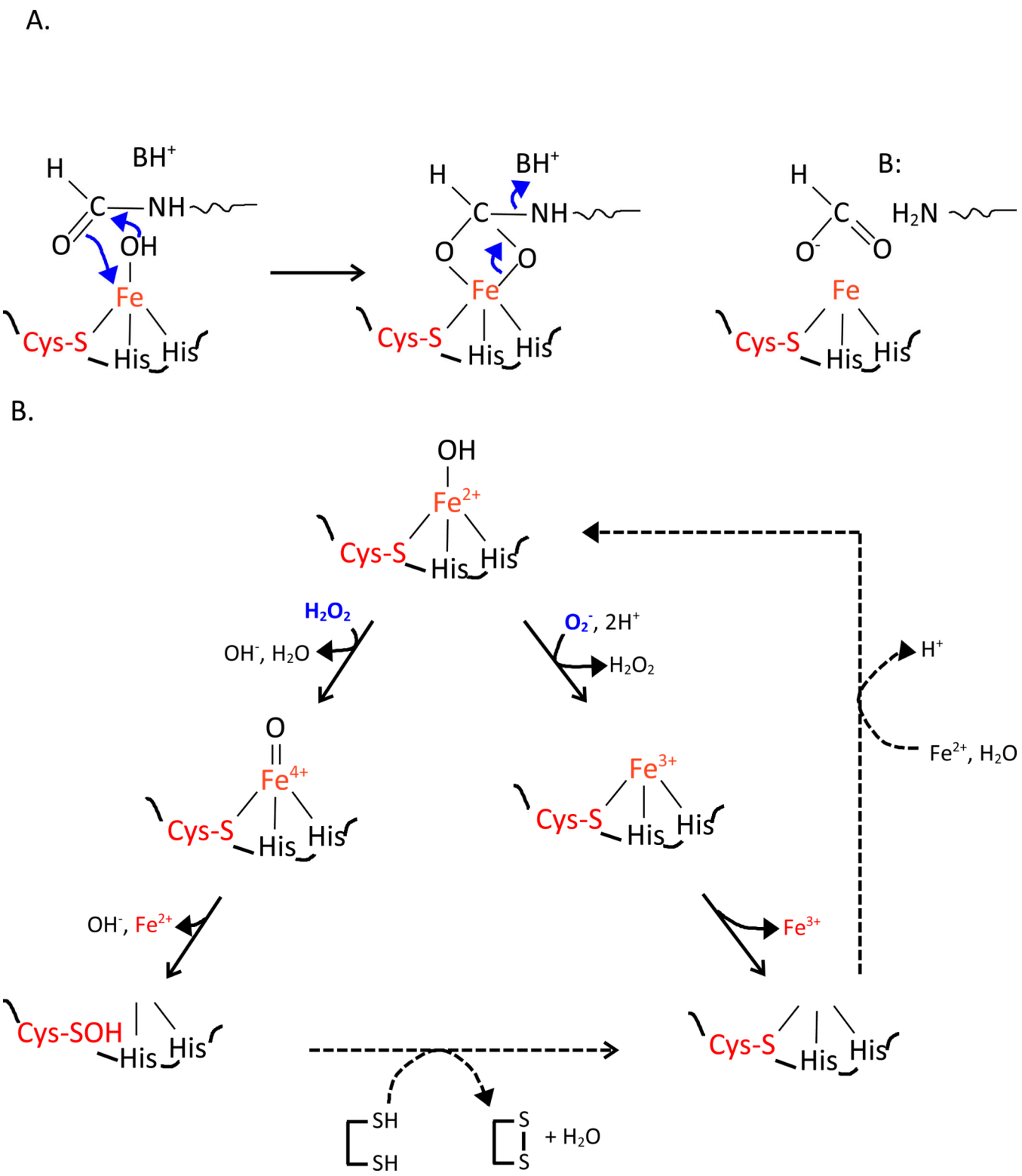

Fig. 4. The role and oxidative vulnerability of mononuclear iron enzymes

Hydrogen peroxide and superoxide also diminish the activity of mononuclear iron enzymes, which use single iron atoms as prosthetic groups. (a) Peptide deformylase is presented as an

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example of this enzyme class. The cationic iron atom of peptide deformylase both activates a water molecule to provide a strong hydroxyl nucleophile (left) and stabilizes the negatively charged oxygen atom of the reaction intermediate (center)[141]. BH^+ represents the enzymic proton donor that ultimately cleaves the carbon-nitrogen bond. (b) In the left pathway, oxidation of the mononuclear iron enzyme by H_2O_2 generates a transient ferryl species (Fe^{4+}=O) that is then quenched by a coordinating cysteine residue. Since a sulphenic species (-SOH) is the ultimate product[76], it seems likely that a thiyl radical electron is transferred to the departing iron atom. The right pathway shows oxidation by superoxide, which generates a ferric iron species (Fe^{3+}) that dissociates. The dashed black lines show that the activity of the superoxide-generated apoprotein can be restored by simple remetallation, although mismetallation of these enzymes by zinc can progressively diminish activity. Reactivation of the H_2O_2-damaged enzyme requires sulphenic reduction prior to remetallation (dashed grey lines).

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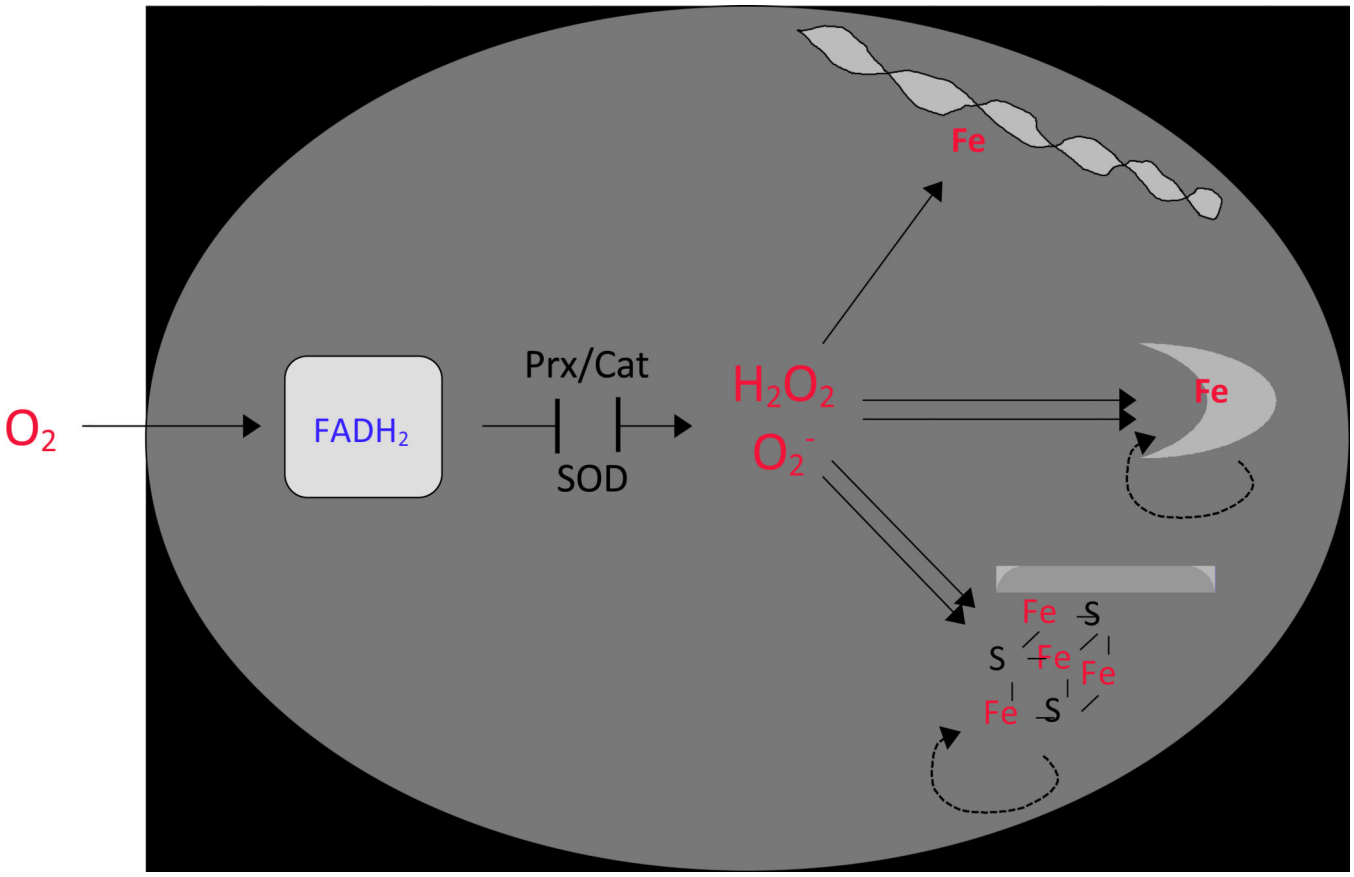

Fig. 5. Overview of *E. coli* damage by reactive oxygen species

The autoxidation of redox enzymes leads to continuous H_2O_2 and O_2^- formation. Catalases (Cat), peroxidases (Prx) and superoxide dismutases (SOD) minimize the accumulation of these two oxidants. Nevertheless, both species damage [4Fe-4S] dehydratases and mononuclear iron enzymes. The disabled enzymes are continuously repaired, so that their steady-state activities represent the balance between damage and repair processes. H_2O_2 also reacts directly with the pool of unincorporated ferrous iron, which loosely associates with biomolecules, including DNA. The resultant hydroxyl radicals damage DNA, requiring the action of repair enzymes. The basal defenses of the cell keep the rates of these injuries low enough that growth and viability are not noticeably affected. However, when H_2O_2 and/or superoxide-generating redox compounds enter the cell, the intracellular levels of these oxidants rise; consequently, the vulnerable enzymes become predominantly disabled and metabolic pathways fail. Under these conditions, the induction of OxyR- and SoxRS-directed defence regulons are essential for cell recovery.