SUPEROXIDE STRESS IN *ESCHERICHIA COLI*

BY

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DISSERTATION

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ABSTRACT

Since its discovery in 1969, superoxide (O$_2^-$) has been recognized as a primary source of oxidative stress. However, in the last 40 years, the only cellular target of O$_2^-$ that has been identified is a subfamily of [4Fe-4S] dehydratases. The inactivation of those dehydratases can only explain part of its toxic effects, which implies the existence of other unknown cellular targets. We have recently identified a new class of cellular targets of O$_2^-$: non-redox mononuclear iron enzymes. These enzymes employ a ferrous iron atom [Fe(II)] as a solvent-exposed cofactor. Four such enzymes -- threonine dehydrogenase (Tdh), ribulose-5-phosphate 3-epimerase (Rpe), peptide deformylase (PDF), and DAHP synthase -- were shown to be inactivated in the *Escherichia coli* strain which lacks the ability to scavenge O$_2^-$ (denoted as SOD$^{-}$ mutant). Moreover, the inactivation of DAHP synthase, the first enzyme in the aromatic amino acid biosynthetic pathway, could partially explain the aromatic amino acid auxotrophy of SOD$^{-}$ mutants. All four enzymes were purified and were demonstrated to be rapidly inactivated by O$_2^-$ *in vitro*. The inactivation rate constants were comparable to those with which O$_2^-$ reacts with [4Fe-4S] dehydratases. It was initially a puzzle how O$_2^-$ could damage those iron enzymes *in vivo*, since unlike H$_2$O$_2$ which can form a ferryl radical with the catalytic Fe(II) and damage the polypeptide, O$_2^-$ is not able to do so. In fact, we found that Tdh and Rpe isolated from the O$_2^-$-stressed stain were metallated with Zn(II) rather than with Fe(II). Therefore, we propose that O$_2^-$ oxidizes Fe(II), the oxidized Fe(III) dissociates from the enzyme, and Zn(II) gets the chance to bind. Since Zn(II) binds to these enzymes much more tightly than does iron, it traps the enzymes in the mismetallated form which are much less efficient in catalysis.
O₂⁻-sensitive [4Fe-4S] dehydratases and mononuclear iron enzymes are present throughout metabolism. Therefore, a number of bacteria and plants exploit this vulnerability: they excrete antimicrobial compounds known as redox-cycling drugs to elevate the production of O₂⁻ in their competitors. When *E. coli* is exposed to these compounds, its SoxR transcription factor is activated by oxidation of its [2Fe-2S] cluster. O₂⁻ was initially thought to be the activator of SoxR, because the O₂⁻-scavenging enzyme – superoxide dismutase (SOD) is a member of the SoxRS regulon. However, we found that abundant O₂⁻ did not effectively activate SoxR in an SOD⁻ mutant, that overproduced SOD could not suppress activation by redox-cycling drugs, and that redox-cycling drugs were able to activate SoxR in anaerobic cells as long as alternative respiratory acceptors were provided. Thus O₂⁻ is not the signal that SoxR senses. Indeed, redox-cycling drugs directly oxidized the cluster of purified SoxR in *vitro*, while O₂⁻ did not. Redox-cycling drugs also caused cellular toxicity independent of the production of O₂⁻, as they poisoned *E. coli* under anaerobic conditions, in part by oxidizing [4Fe-4S] dehydratases. SoxRS affects the expression of nearly 100 genes, most of which do little to ameliorate O₂⁻ toxicity. Instead, they focus upon reducing the intracellular levels of redox-cycling drugs. Thus it is physiologically appropriate that the SoxR protein directly senses redox-cycling drugs rather than O₂⁻.
To the memory of my mother and to my family
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1.1 Discovery of superoxide

Superoxide (O$_2^-$) is generated by the univalent reduction of molecular oxygen. The first piece of evidence that O$_2^-$ exists within cells came from the work of McCord and Fridovich. They were examining the \textit{in vitro} reaction in which xanthine oxidase converts xanthine to urate and hydrogen peroxide (H$_2$O$_2$). The reaction mixture was able to reduce cytochrome c (82). Initially, they thought that xanthine oxidase could directly reduce cytochrome c. However, the reaction mixture did not reduce cytochrome c under anaerobic conditions. Therefore, they proposed that during the oxidation of xanthine by xanthine oxidase, an unstable form of oxygen – the superoxide anion (O$_2^-$) – was generated, and that O$_2^-$ reduced cytochrome c. Since O$_2^-$ is highly unstable, it was first suggested that O$_2^-$ made in this reaction was bound to xanthine oxidase. But their later experiments confirmed that O$_2^-$ was not bound to xanthine oxidase and free O$_2^-$ reduced cytochrome c in solution (83).

\[
\text{xanthine oxidase}
\]

\[
(1) \quad \text{xanthine} + \text{O}_2 \rightarrow \text{urate} + \text{O}_2^- + \text{H}_2\text{O}_2
\]

\[
(2) \quad \text{cytochrome c (ox)} + \text{O}_2^- \rightarrow \text{cytochrome c (red)} + \text{O}_2
\]

McCord and Fridovich also observed that cytochrome c reduction by xanthine oxidase could be inhibited by carbonic anhydrase and myoglobin preparations from bovine and human erythrocytes (28, 82). This implied that “something” in their bovine and human erythrocytes preparations scavenged O$_2^-$. The following dismutation reaction was proposed:
Eventually, the "contaminating" dismutase activity was separated from the carbonic anhydrase activity in the bovine erythrocytes preparations (83). The blue-green protein purified from bovine erythrocytes with the function of dismuting $O_2^-$ was then named superoxide dismutase (SOD). The first identified SOD was a copper-containing enzyme, as indicated from its blue-green color. The existence of a $O_2^-$–specific enzyme within bovine erythrocytes gave the first indication that $O_2^-$ is produced within cells. Later, other types of SODs with different transition metals in the active site were discovered. The apparent absence of SODs from obligate anaerobes led to the proposal that scavenging $O_2^-$ may be critical to the survival of aerobic organisms.

### 1.2 Superoxide dismutases

#### 1.2.1 Superoxide dismutase reaction.

Superoxide dismutases (SODs) are metalloenzymes that require a transition metal at the active site for the dismutation reaction of superoxide into hydrogen peroxide and molecular oxygen.

$$\text{Me (ox) + } O_2^- \longrightarrow \text{Me (red) + } O_2 $$

$$\text{Me (red) + } O_2^- + 2H^+ \longrightarrow \text{Me (ox) + } H_2O_2 $$

Net reaction: $O_2^- + O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2$

SODs are so far the most efficient enzymes, and they catalyze $O_2^-$ dismutation at diffusion-limited rates ($k=2\times10^9 \text{ M}^{-1} \text{ s}^{-1}$) (83). $O_2^-$ can dismute spontaneously, which
occurs at a rate of $10^5 \text{ M}^{-1} \text{s}^{-1}$ (30). The spontaneous dismutation of $O_2^-$ is a second-order reaction ($O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$), therefore it slows dramatically as $O_2^-$ concentration decreases. Cytoplasmic SODs keep the steady-state concentration of $O_2^-$ at $\sim 10^{-10}$ M. In cells lacking SODs, the steady-state $O_2^-$ concentration was estimated to be up to 1000-fold higher (50).

### 1.2.2 Four classes of superoxide dismutases.

Based on the transition metal in the active site, there are four classes of SODs: manganese (MnSOD), iron (FeSOD), copper-zinc (Cu/ZnSOD), and Ni (NiSOD). MnSODs and FeSODs are mostly located in the mitochondria or chloroplasts of higher eukaryotes, and in the cytoplasm of bacteria (7, 38). Their structures and catalytic properties have been well established (55). In most species, both enzymes form a homodimer of two 23 kDa subunits, with each containing one metal atom at the active site. The active site is strictly conserved with three histidines and one aspartate to ligand the transition metal, which undergoes oxidation-reduction cycles for the dismutation of $O_2^-$. The third major class of SODs is the Cu/Zn SOD, which has both copper and zinc in the active site (104). Cu is the metal that undergoes redox state change in the dismutation reaction. Cu/Zn SODs exist in the cytosol or in the intermembrane space of mitochondria and chloroplasts in eukaryotes; and they are found in the periplasms of some bacteria, including *E. coli* (29). NiSOD has only been found in some *Streptomyces* species and one marine *Cyanobacterium* (73).
1.2.3 Regulation of cytoplasmic superoxide dismutases in *E. coli*.

*E. coli* has two cytoplasmic SODs – MnSOD, encoded by the gene *sodA*; and FeSOD, encoded by the gene *sodB* (59, 120). The presence of two SODs in the same cellular compartment suggests that they might be differently regulated.

In fact, *sodB* is constitutively expressed under both anaerobic and aerobic conditions, and it is positively regulated by Fur through RyhB (11, 20, 21, 23). Fur stands for Ferric Uptake Regulator. It senses the cytosolic free iron levels and regulates gene expression accordingly (4, 37, 81). When cellular iron levels are high, Fur binds to Fe(II) and shuts down expression of RyhB small RNA, which would otherwise bind and trigger degradation of the mRNAs of FeSOD. Therefore, under iron replete conditions, FeSOD is the more abundant SOD.

*sodA* expression is intensively regulated. Its expression is activated under aerobic conditions due to the loss of repression by Fnr and ArcA (41, 105). Fnr is active under anaerobic conditions, and it blocks *sodA* transcription. Under aerobic conditions, Fnr is inactivated by molecular oxygen and therefore is unable to repress *sodA* transcription (103). Phosphorylated ArcA is known to repress a number of genes that are unnecessary for anaerobic growth (54). The phosphorylated ArcA binds to the promoter region of *sodA* and blocks its transcription. The *sodA* gene is also a member of the *fur* regulon. When cellular iron levels are high, Fur binds to Fe(II) and represses *sodA* expression; while under iron depletion conditions, MnSOD expression is induced, presumably to compensate for the loss of FeSOD activity (23, 106). MnSOD is also induced under stress conditions, such as exposure to redox-cycling drugs, by SoxRS. The SoxRS response will be discussed in more detail in section 1.7.
1.3 Sources of superoxide

1.3.1 Internal sources of superoxide

SOD-deficient cells exhibit growth defects aerobically (11), indicating that $O_2^-$ is constantly generated during aerobic growth. Being a charged molecule, $O_2^-$ cannot cross membranes. Therefore, endogenous $O_2^-$ is produced only when molecular O$_2$ adventitiously steals an electron from cellular components. The formation of $O_2^-$ requires that the cellular component pass one electron to molecular O$_2$, and the reductant itself must be able to stabilize its unpaired electron left behind, or have only one electron to donate. Therefore, some universal cellular reductants, such as NADH, cannot reduce molecular O$_2$, since NADH is an obligate 2-electron donor (71). In contrast, flavosemiquinones, thyl radicals, ubiquinones, and menaquinones are stable, which make them potential cellular reductants for molecular O$_2$. In addition, transition metals, such as those found in enzyme metal centers, can also serve as univalent reductants. Therefore, enzymes using these co-factors are candidates for producing endogenous O$_2^-$. In E. coli, flavoenzymes in the respiratory chains, including NADH dehydrogenase II and fumarate reductase, are capable of generating O$_2^-$ at substantial rates in vitro (49, 51, 85). NADH dehydrogenase II functions in the aerobic respiratory chain, and it employs a flavin co-factor for electron transport. This enzyme is also accessible to molecular O$_2$ and generates roughly 4 O$_2^-$ molecules per 10,000 electrons that pass through the protein in vitro (51). The rate of O$_2^-$ production by the respiratory chain thereby has been estimated to be about 5 µM per second. Fumarate reductase is a member of the anaerobic respiratory chain. It has been shown to generate O$_2^-$ both in vivo and in vitro. Similarly, its flavin co-factor is the site of O$_2^-$ production (49, 85, 86). In
addition, two other flavin-dependent enzymes – sulfite reductase and NadB have been identified as minor sources of $O_2^-$ in the cytoplasm (66, 84). A general model of endogenous $O_2^-$ formation is shown in Fig. 1.1.

As is stated, a number of flavoproteins have been shown to be able to make $O_2^-$ in vitro. However, our lab showed that $O_2^-$ production was not lessened in cells lacking NADH dehydrogenase II, which in vitro was the most autoxidizable component of the electron transport chain (66). In fact, fumarate reductase is so far the only cellular component that has been identified to generate $O_2^-$ in vivo (86). The predominant sources of endogenous $O_2^-$ have not been determined yet.

1.3.2 External sources of superoxide.

There are also environmental sources of $O_2^-$. Certain organisms, including plants and bacteria, can produce and secrete compounds known as redox-cycling drugs to inhibit the growth of their competitors (14, 53). For example, plumbagin was secreted by the plant Plumbago (112); and phenazines are made by bacteria such as Pseudomonas, Streptomyces, and Pantoea agglomerans (111). These drugs can enter the cells and steal single electrons from the reduced flavins or metal centers of redox enzymes. The reduced drug can then pass the electron to molecular $O_2$, generating $O_2^-$ (Fig. 1.2). The rate of $O_2^-$ formation by such chemicals can exceed the normal rate of $O_2^-$ production by several orders of magnitude (39).

During bacterial infections, activated macrophages and phagocytes deliberately generate $O_2^-$ to attack the bacterial invaders. This is called the oxidative burst. It is accomplished by the host enzyme NADPH oxidase, which is localized to the phagosome
NADPH oxidase is a membrane-associated enzyme containing a flavin co-factor. It releases reactive oxygen species, including $O_2^-$ and $H_2O_2$, into the membrane vesicle that contains the bacterial invaders. Invading organisms have been shown to require superoxide scavenging enzymes in the periplasm to be able to survive in their hosts (67, 68).

1.4 Cytoplasmic targets of superoxide

In 1986, Carlioz and Touati showed that *E.coli* strains lacking both cytosolic superoxide dismutases (SOD$^-$ mutants) are unable to grow aerobically without supplements of branched-chain, aromatic and sulfurous amino acids (11). The branched-chain amino acid auxotrophy of SOD$^-$ mutants has been attributed to the damage of two [4Fe-4S] cluster dehydratases, dihydroxyacid dehydratase and isopropylmalate isomerase, in the branched-chain biosynthetic pathways (24-26).

1.4.1 [4Fe-4S] cluster dehydratases.

Fe-S clusters are commonly used enzyme cofactors. In the subfamily of [4Fe-4S] cluster dehydratases, three of the four iron atoms are coordinated to the sulfur atoms of the Cys residues in the protein polypeptide, and the forth iron atom is exposed to solvent (Fig. 1.3). This exposed iron binds substrate and is where catalysis takes place (72). With a maximum coordination number of six, iron has tremendous geometric flexibility in binding ligands. In the case of dehydratases, iron switches from the tetrahedral to the octahedral conformation upon substrate binding. The process does not involve significant activation energy, since no bonds are broken (48). During the course of
catalysis, the cationic iron also acts as a Lewis acid to help pull away the anionic hydroxyl group, while a base withdraws a hydrogen atom from the proximal carbon. This results in a net dehydration reaction. Unlike in redox-based reactions, the solvent-exposed iron does not change its redox state, but instead it provides a local positive charge to assist in catalysis, stabilizes the carboxylate that serves as an electron sink, and helps in substrate binding. Aconitase, fumarase, 6-phosphogluconate dehydratase, dihydroxyacid dehydratase, and isopropylmalate isomerase are well studied [4Fe-4S] cluster dehydratases.

1.4.1.1 Vulnerability of [4Fe-4S] cluster dehydratases to superoxide

Since the [4Fe-4S] cluster in dehydratases is exposed to solvent, it may be naturally accessible to small oxidants, such as $O_2^-$. Flint and coworkers were able to demonstrate that solvent-exposed [4Fe-4S] clusters are highly sensitive to $O_2^-$, and the rates of inactivation were measured to be at $10^6 - 10^7$ M$^{-1}$s$^{-1}$ (26). The proposed inactivation mechanism (shown in Fig. 1.3) is that $O_2^-$ oxidizes the solvent-exposed [4Fe-4S]$^{2+}$ cluster (Reaction 1), resulting in release of free iron and an inactive [3Fe-4S]$^+$ cluster (Reaction 2). The free ferrous iron causes further problems to the cells.

$$\text{[4Fe-4S]}^{2+} + O_2^- + 2H^+ \longrightarrow \text{[4Fe-4S]}^{3+} + H_2O_2 \quad \text{(Reaction 1)}$$

$$\text{[4Fe-4S]}^{3+} \longrightarrow \text{[3Fe-4S]}^+ + Fe^{2+} \quad \text{(Reaction 2)}$$

$H_2O_2$ damages [4Fe-4S] cluster dehydratases, in a manner analogous to the Fenton reaction. The rate of inactivation by $H_2O_2$ is comparatively slow at $10^3 - 10^4$ M$^{-1}$s$^{-1}$ (57). However, the intracellular $H_2O_2$ concentration is $\sim 10^{-8}$ M (99), which is about 100-fold higher than $O_2^-$, therefore the overall impact of $H_2O_2$ and $O_2^-$ on the dehydratases will be
quite similar. The *E. coli* Hpx· mutants lacking the ability to scavenge H₂O₂ also exhibit branched-chain amino acid auxotrophy, which has been attributed to the inactivation of the two [4Fe-4S] cluster dehydratases in the branched-chain biosynthetic pathways. The following reactions have been suggested to take place upon cluster damage (57):

\[
[4\text{Fe}-4\text{S}]^{2+} + \text{H}_2\text{O}_2 \rightarrow [4\text{Fe}-4\text{S}/\text{O}]^{2+} + \text{H}_2\text{O}
\]

\[
[4\text{Fe}-4\text{S}/\text{O}]^{2+} + \text{H}^+ \rightarrow [3\text{Fe}-4\text{S}]^+ + \text{Fe}^{3+} + \text{OH}^-
\]

To defend against H₂O₂, *E. coli* turns on its OxyR response. OxyR is a LysR-type regulator. H₂O₂ activates OxyR by oxidizing its sulfhydryl to a disulfide bond (13, 74, 121). H₂O₂ oxidizes OxyR at an extremely high rate of \(10^7\) M⁻¹ s⁻¹, and it has been shown that less than 1 µM H₂O₂ is enough to effectively activate OxyR. DNA microarray data showed that OxyR induces the expression of at least 20 genes (122) (Table 1.1). The products encoded by OxyR-regulated genes include: H₂O₂-scavenging enzymes AhpCF (peroxidase) and KatG (catalase), proteins involved in iron homeostasis, such as Fur and Dps, manganese transporter MntH, Fe-S cluster assembly system Suf, *et al.* These gene products work cooperatively to limit the amount of H₂O₂ inside cells, and to limit and repair the damages caused by it.

Since its discovery in 1969, the only cellular target of O₂ that has been identified is the [4Fe-4S] cluster dehydratases. Inactivation of dihydroxyacid dehydratase and isopropylmalate isomerase results in the branched-chain auxotrophy in SOD· mutants. Inactivation of aconitase and fumarase prevents these mutants to catabolize carbon sources that are normally assimilated by the TCA cycle. SOD· mutants are also auxotroph for aromatic and sulfur-containing amino acids. However, there are no such
[4Fe-4S] cluster dehydratases in their biosynthetic pathways, indicating the existence of other unknown cellular targets of O$_2^\cdot$.

1.4.1.2 Repair of [4Fe-4S] cluster dehydratases

The rate constant for inactivation by O$_2^\cdot$ is so high (≈10$^6$–10$^7$ M$^{-1}$ s$^{-1}$) that it is likely that even wild-type cells contain enough superoxide to continually damage these enzymes. In response, bacterial cells have acquired the ability to repair the damaged clusters. In fact, when superoxide-stressed cells were returned to anaerobiosis, the damaged dehydratases quickly restored their activities in the absence of new protein synthesis. The half-time for cluster repair is about 5 min (31, 61). Thus, during aerobic growth the steady-state activity of these enzymes reflects the dynamic balance between enzyme oxidation and repair.

The mechanism of repair remains unknown. Conversion of a [3Fe-4S]$^{1+}$ cluster back to the active [4Fe-4S]$^{2+}$ form requires both reduction and metallation. Reduction probably happens first so that the unstable [4Fe-4S]$^{3+}$ intermediate may be avoided. The damaged clusters could disintegrate beyond the [3Fe-4S]$^{1+}$ form in vivo when stress is prolonged (19). In that case, cluster repair might utilize the same pathway that originally assembles the cluster in newly synthesized proteins. This process is accomplished in E. coli by proteins in either the Isc system or the Suf system (6, 27). However, it has been shown that: 1) only the iscS mutant exhibited a substantial defect in cluster repair, while all isc genes are important for de novo cluster assembly; 2) E. coli mutants lacking Suf proteins repaired clusters at normal rates. Therefore, the
mechanism of cluster repair under mild oxidative stress condition is distinct from that of  
de novo assembly.

Recent studies have suggested that YggX, a member of the SoxRS regulon, is  
inviolved in cluster repair (101). The yggX null mutation slowed the recovery of  
dehydratase activities in SOD\atherss mutants upon return to anaerobiosis. Also,  
overproduction of yggX enabled SOD\atherss mutants to fully restore dehydratase activities and  
at rates similar to the wild type strain. The repair mechanisms of YggX remain unclear.  
Another protein that might play a role in cluster repair is the di-iron protein YtfE (58).  
ytfE is not under control of either OxyR or SoxRS. Its expression was found to be highly  
stimulated by nirosative stress and iron starvation. It has been shown that the damage  
of the [4Fe-4S]\ superscript{2+} clusters of aconitase B and fumarase A caused by exposure to  
hydrogen peroxide and nitric oxide occured at higher rates in the absence of ytfE. The  
ytfE null mutation also abolised the recovery of aconitase and fumarase activities,  
which was observed in wild type E. coli once the stress was scavenged. Upon the  
addition of purified holo-YtfE protein to the mutant cell extracts, the enzymatic activities  
of fumarase and aconitase were recovered at rates similar to the wild type strain.  
However, researchers in our lab failed to reproduce the effects of YtfE in Fe-S cluster  
repair.

1.4.2 DNA.

It has been shown that growing E. coli cells exposed to H\atherss\subscript{2}O\atherss\subscript{2} \textit{in vivo} exhibit DNA  
damage, and that DNA-repair mutants are killed rapidly (12, 15, 52). This type of  
damage is mediated by Fenton chemistry, which is shown below. This idea is strongly
supported by two findings: (1) cell-permeable iron chelators are able to prevent DNA damage in the presence of H$_2$O$_2$ (52); (2) DNA damage proportionally rises with conditions that elevate intracellular free iron levels.

Fenton chemistry: $\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{HO}^•$

The highly reactive HO• thus generated attacks DNA, leading to the formation of a variety of adducts, such as 8-hydroxyguanine (10), and also DNA strand cleavage (18, 46). The rate constant of Fenton chemistry is 5000–20,000 M$^{-1}$s$^{-1}$ (92). This rate is fast enough for less than 1 µM H$_2$O$_2$ to create lethal amounts of DNA damage in cells.

McCord and Fridovich observed that xanthine oxidase could damage DNA. When catalase, SOD or the iron chelator EDTA was included in the reaction mixture, DNA was protected. The straightforward explanation was that H$_2$O$_2$, O$_2^-$ and iron contributed to DNA damage. Therefore, it was first hypothesized that O$_2^-$ contributed to oxidative DNA damage in vivo by acting as the reductant of Fe(III), which generated Fe(II) for the Fenton chemistry. This hypothesis was reinforced by the finding that the SOD$^-$ mutants exhibited 10- to 40-fold higher rates of spontaneous mutagenesis than did the wild type (22), and that these mutants were more sensitive to H$_2$O$_2$ killing (52).

However, if O$_2^-$ were the sole reductant, its presence would be required to cause DNA damage during H$_2$O$_2$ challenge. In fact, H$_2$O$_2$ damages DNA even under anaerobic conditions, where O$_2^-$ cannot be made. Moreover, when SOD$^-$ mutants were grown aerobically and made anaerobic 20 minutes prior to H$_2$O$_2$ challenge, they still showed a higher level of sensitivity to H$_2$O$_2$ killing than did wild type cells (60). Later, it was demonstrated that the ability of O$_2^-$ to increase the intracellular free iron levels was the actual reason for the accelerated oxidative DNA damage in SOD$^-$ mutants (62). In other
words, $O_2^*$ does not directly damage DNA; rather, it drives the Fenton chemistry by elevating the available free iron levels.

1.5 Iron metabolism

1.5.1 The origin of iron usage in biological systems.

Life evolved on the Earth around 3.8 billion years ago in an anaerobic environment. Ferrous iron and sulfur were abundant, and they were recruited in the formation of Fe-S clusters functioning in various proteins (48). Ancient organisms employed these proteins to conduct difficult and biologically important reactions. About 2.5 billion years ago, molecular oxygen, a by-product of oxygenic photosynthesis, started to accumulate in the atmosphere (65, 69). This created two threats to cluster-dependent proteins: (1) oxygen oxidizes ferrous iron in the environment, making iron unavailable to cells. (2) Reactive oxygen species oxidize exposed Fe-S clusters and make them inactive in catalysis. Some microbes responded by retreating to anaerobic habitats; others evolved complex schemes to protect, repair or bypass the labile Fe-S cluster proteins. Nowadays, however, with a few exceptions, organisms are still dependent on iron for a wide variety of cellular processes (100).

1.5.2 Regulation of iron uptake, storage and usage.

To meet their iron requirement in aerobic environments, microbes have evolved a complicated iron-import system. In this system, they produce and secrete soluble organic molecules called siderophores to bind iron and leach it off mineral precipitates.
Since the resultant iron-siderophore complexes are too big to pass through outer membrane porins, bacteria evolved specific iron-siderophore transporters in their outer membranes (117). When inside the cell, iron is released from the complexes by siderophore hydrolysis (9). This iron delivery system is remarkably expensive – it involves synthesis of specific secreted molecules and cellular components, and it is single-turnover.

Due to the high cost of the siderophore system, microbes tend to employ simpler iron import systems when their habitats are relatively iron replete. They inactivate their siderophore system through the activation of Fur. Fur stands for Ferric Uptake Repressor. It senses intracellular free iron levels and regulates gene expression accordingly (Fig. 1.4). Fur functions as a dimer in vivo, with each monomer binding a ferrous iron atom. When iron is abundant, Fur binds iron and represses the expression of siderophore biosynthesis and uptake genes (89). Metallated Fur also blocks transcription of the small RNA RyhB, which would otherwise bind and trigger degradation of mRNAs of a number of iron-containing proteins. RyhB-controlled proteins include Fe-S cluster enzymes succinate dehydrogenase and NADH dehydrogenase I, iron-storage protein ferritin, and iron-containing SOD (81). In iron-replete habitats, this control system enables microbes to synthesize iron-containing proteins to support healthy growth, and to store excess iron for future use.

When cells are starved for iron, Fur is demetallated, and iron uptake genes are de-repressed. Meanwhile, RyhB is made to suppress the synthesis of iron storage proteins and Fe-S enzymes, such as NADH dehydrogenase I and succinate dehydrogenase (81). These repressed enzymes are not essential. By suppressing their synthesis, limited iron is directed to the iron enzymes that provide the greatest benefit to cells.
1.5.3 Iron-dependent biochemical reactions.

Iron can participate in a vast variety of biochemical reactions inside cells, including redox and non-redox based ones. The versatility of iron stems from its chemical properties. Iron is a transition element with an unfilled $d$-orbital; therefore, it exists in a wide range of oxidation states, $-2$ to $+6$, although $+2$ and $+3$ are the most common. Its multiple oxidation states enable iron to participate in various electron transfer reactions, and to bind to different numbers of ligands.

1.5.3.1 Redox based reactions

In this type of enzyme reaction, iron changes its redox status during catalysis. Examples of such enzymes are heme-based enzymes, such as catalase; and redox mononuclear iron enzymes, such as FeSOD.

Catalase is one of the most well-known examples of heme-based enzymes. Catalase scavenges $\text{H}_2\text{O}_2$ in a two-step reaction to generate $\text{H}_2\text{O}$ and $\text{O}_2$ (8):

\[
(1) \quad \text{H}_2\text{O}_2 + \text{Fe(III)}-\text{E} \rightarrow \text{H}_2\text{O} + \text{O}=\text{Fe(IV)}-\text{E}. (+) \\
(2) \quad \text{H}_2\text{O}_2 + \text{O}=\text{Fe(IV)}-\text{E}. (+) \rightarrow \text{H}_2\text{O} + \text{Fe(III)}-\text{E} + \text{O}_2
\]

$\text{Fe}()\text{-E}$ represents the iron center of the heme group attached to the enzyme. Catalase has one of the highest turnover numbers of all enzymes, owing to the ability of iron to convert quickly between two oxidation states.

In redox mononuclear iron enzymes such as FeSOD, iron cycles between the ferric (+3) and ferrous (+2) forms (113) (reviewed in detail in section 1.2.). Notably, these enzymes function only when iron is bound at their active sites, and no other metals can be used as substitutes.
1.5.3.2 Non-redox based reactions

In this type of enzyme reactions, iron does not go through a change in its oxidation states. One such example is the [4Fe-4S] cluster dehydratases. As stated before, three of the four iron atoms are coordinated to the sulfur atoms of the Cys residues in the protein polypeptide, and the forth iron atom is solvent-exposed. This exposed iron binds substrate and is the site of catalysis (72). Upon substrate binding, iron switches from the tetrahedral to the octahedral conformation. This solvent-exposed iron also assists in catalysis by acting as a Lewis acid to pull away a hydroxyl group, while a base withdraws a hydrogen atom from the proximal carbon. During catalysis, this solvent-exposed iron does not go through a change in its oxidation states.

Dehydratases are not the only enzymes that use iron in a non-redox manner. Recent studies in our lab have identified several non-Fe/S enzymes that use iron in vivo in a non-redox way. These enzymes are ribulose-5-phosphate 3-epimerase, peptide deformylase, threonine dehydrogenase and cytosine deaminase. They utilize a single ferrous iron as the solvent-exposed co-factor, and therefore belong to the family of enzymes termed non-redox mononuclear iron enzymes. Fig. 1.5 shows the crystal structure of E. coli peptide deformylase as an example of the non-redox mononuclear iron enzymes. The overall role of iron in these enzymes is similar to the one in dehydratases – to provide a local positive charge and to help with substrate binding.

1.5.4 Metal usage in redox based and non-redox based mononuclear enzymes.

As in the case of Fe, Mn, Co, Ni, and Zn are also first-row transition metals. They share similar chemical characteristics – they have partially filled d-orbitals; they have
similar size and valence; and they have a ligand coordination number up to six. In fact, it has been shown that the non-redox mononuclear iron enzymes can be activated by transition metals other than iron in vitro, and in some cases in vivo. For example, ribulose-5-phosphate 3-epimerase can be activated by Fe$^{2+}$, Mn$^{2+}$ and Co$^{2+}$ in vitro, and manganese import ensures the continued function of ribulose-5-phosphate 3-epimerase during hydrogen peroxide stress or iron starvation (102). Similarly, peptide deformylase can be activated by Fe$^{2+}$, Mn$^{2+}$, Ni$^{2+}$ and Co$^{2+}$ in vitro, and E. coli defends it during hydrogen peroxide stress by inducing the manganese importer MntH (2). It is not surprising that other transition metals can activate those non-redox mononuclear iron enzymes, since in such enzymes the metal does not change its redox state but rather provides a local positive charge and helps in substrate binding.

Unlike non-redox mononuclear enzymes, redox-based enzymes require the use of their native metal. Superoxide dismutases (SODs) are well-studied redox mononuclear enzymes. There are four isozymes of SODs, each employing its unique metal. For example, no other metals can replace iron in the iron-containing superoxide dismutases (FeSOD). Even though manganese can bind at the active site of FeSOD, the resultant enzyme is not catalytically active (109). Similarly, the redox binuclear enzymes - ribonucleotide reductases (Nrd) - also have strict requirement for metal usage (80). NrdAB can be activated by both manganese and iron, although it is substantially more efficient with iron than with manganese. Oppositely, NrdEF is substantially more efficient with manganese than with iron.
1.5.5 *in vivo* metal specificity determination.

As is stated in sec. 1.5.4, transition metals share similar properties and may functionally replace one another in the non-redox mononuclear enzymes *in vitro*. In addition, it has been shown that metals in non-redox mononuclear enzymes have high dissociation rates *in vitro* (2, 102). Therefore, an apparent challenge cells have to face is to deliver and maintain the correct metals for those metalloenzymes. Logically, there are three key factors that contribute to populating enzymes with correct metals: (1) the availability of metals inside cells; (2) the affinities of different metals for the enzyme active site; and (3) the role of metallochaperones and other cellular components in delivering and maintaining the correct metal at the enzyme active site.

An extreme example that metal availability determines enzyme mettallation comes from cyanobacteria. In these microbes, correct mettallation is ensured by having different metalloproteins fold within different cellular compartments (108). The cytoplasm is manganese rich and copper poor, so proteins that are supposed to bind manganese will fold in the cytoplasm; while the periplasm is copper rich and manganese poor, so proteins that are supposed to bind copper will fold in the periplasm. This strategy successfully prevents mismetallation of proteins.

In *E. coli*, EPR analysis showed that the concentration of intracellular unincorporated iron is 20–50 μM, and it exists almost entirely in the ferrous form (118). Such a ferrous iron pool is likely to be sufficient to activate any enzyme with a high affinity mononuclear site. Zinc is relatively abundant in *E. coli*, and its intracellular concentration is mainly regulated by the Zn(II) efflux pump ZntA (97). Total zinc content in *E. coli* has been estimated to be ~ 600 μM, including unincorporated zinc as well as zinc coordinated with proteins (70). However, the intracellular level of free zinc is hard to measure, and it has
been estimated to be in the range of 10 μM based on $K_m$ of ZntA (97). Manganese is typically scarce (<10 μm) (3). E. coli typically does not use cobalt, and it lacks a dedicated cobalt transport system. Nickel is imported only under anaerobic conditions when the nickel-dependent hydrogenase is induced (98). Copper exists as Cu(I) in the cytoplasm, and its concentration is kept extremely low by three homeostatic systems. CopA is an ATPase that pumps copper from the cytoplasm into the periplasm (96). CueO oxidizes periplasmic Cu(I) to Cu(II) in order to slow its entry into the cytoplasm (34). The Cus system pumps copper from the periplasm back to the extracellular environment (88). This strict control of intracellular copper concentration makes perfect sense, given the fact that copper interferes with iron binding in Fe-S clusters and inactivates [4Fe-4S] cluster dehydratases (79).

It is important to note that intracellular metals tend to associate with metabolites and cell surfaces, due to their positive charges. However, it is not yet understood how metals are retrieved and delivered from their associated metabolites and cell surfaces to metalloproteins. Besides, excess metals are often stored. For example, excess iron is stored by ferritin in E. coli (107). The mechanism by which iron is retrieved from ferritin has not been established.

Transition metals have different affinities to the active sites of mononuclear enzymes. According to the Irving-William series, the stabilities of complexes formed by a metal ion follows this order: Mn(II) < Fe(II) < Co(II) < Ni(II) < Cu(II) > Zn(II). E. coli lacks a dedicated cobalt transport system, and it has been shown to carefully control intracellular concentrations of nickel. Copper exists as Cu(I) in the cytoplasm, and its concentration is kept extremely low. Among the three metals that are physiologically relevant to E. coli – Mn$^{2+}$, Fe$^{2+}$, and Zn$^{2+}$ – Zn$^{2+}$ binds several mononuclear enzymes.
much more tightly than does Mn\(^{2+}\) or Fe\(^{2+}\). For example, in the case of ribulose-5-phosphate 3-epimerase, the half time of dissociation is 8 hours for Zn\(^{2+}\), compared to 50 minutes for Fe\(^{2+}\), and 3.5 minutes for Mn\(^{2+}\) (102).

Only a few metallochaperones have been found so far. The copper chaperone CCS was found in yeast and eukaryotes (45), and the nickel chaperone HypA was identified in *Helicobacter pylori* and *Thermococcus kodakaraensis* (116, 119). They are required *in vivo* to deliver the relevant metals to their destinies. It has been suggested that MntS may facilitate manganese delivery to proteins. MntS may act as a manganese chaperone and/or as a small RNA. Little is known about how iron and other transition metals are trafficked inside cells. Proteins including YggX, CyaY and IscA have been suggested to be involved in iron trafficking (1, 33, 115). However, the exact roles of these proteins have not been demonstrated.

1.5.6 Vulnerability of non-redox mononuclear iron enzymes to hydrogen peroxide.

Our lab has demonstrated that four non-redox mononuclear enzymes are charged with iron *in vivo* and therefore are targeted by hydrogen peroxide. These enzymes are ribulose-5-phosphate 3-epimerase, peptide deformylase, threonine dehydrogenase, and cytosine deaminase. *In vivo* these enzymes are damaged in the Hpx\(^{-}\) cells which lack the ability to scavenge H\(_2\)O\(_2\); *in vitro* the purified enzymes are rapidly inactivated by micromolar H\(_2\)O\(_2\) (2, 102). Peptide deformylase and threonine dehydrogenase use a cysteine residue to coordinate the catalytic metal. Upon H\(_2\)O\(_2\) exposure, this cysteine residue is oxidized by the radical generated from the Fenton reaction. When first oxidized to a sulfenic acid (S-OH), it can be reduced and reactivated by cellular
reductants. However, overoxidation of the cysteine residue to sulfenic (S-OOH) or sulfonic (S-OOOH) forms results in irreversible damage of the enzymes (2). Manganese can activate all four enzymes \textit{in vitro} in place of iron, and the manganese-containing enzymes are resistant to H$_2$O$_2$. Manganese supplementation to H$_2$O$_2$-stressed cells protects the enzymes \textit{in vivo}. Therefore, during H$_2$O$_2$ stress, \textit{E. coli} induces MntH, the manganese importer, and Dps, the iron-sequestering protein, to defend these mononuclear iron enzymes.

1.6 Redox-cycling drugs

Redox-cycling drugs are widely present in nature. They are secreted by certain plants and bacteria for antimicrobial and competing purposes. For example, plumbagin, a quinone analogue, was originally isolated from the plant Plumbago; juglone occurs naturally in the leaves, roots, husks, and bark of plants in the Juglandaceae family, particularly the black walnut. Both compounds are effective herbicides that allow the parent plant to dominate a habitat. Phenazines are made by bacteria such as \textit{Pseudomonas, Streptomyces}, and \textit{Pantoea agglomerans}. They exert toxic effects on other bacteria. There are also manmade redox-cycling drugs, and the most commonly used one is methyl viologen (paraquat, PQ). Based on their chemical structures, redox-cycling drugs can be classified into three categories – viologens, quinones and phenazines (Fig.1.6).

Redox-cycling drugs generate O$_2^-$ via one electron oxidative cycling \textit{in vivo}. Each of these drugs can penetrate into the cell interior, where they abstract single electrons from the reduced flavins or metal centers of redox enzymes. The reduced drug can then pass
the electron to molecular oxygen, generating $\text{O}_2^-$. The drug itself gets reoxidized, completing the redox cycle. This redox-cycling behavior can elevate intracellular superoxide formation by orders of magnitude above the usual rate.

1.7 SoxRS response

1.7.1 SoxRS response system (SoxR & SoxS).

Hassan and Fridovich found that manganese-containing SOD (MnSOD) was strongly induced when *E. coli* cells were exposed to redox-cycling drugs in aerobic medium (40). Subsequently, the Demple and Weiss labs independently found that this regulation was mediated by the transcription factor SoxR (35, 110).

SoxR forms a dimer, with each monomer containing a [2Fe-2S] cluster (42). The cluster undergoes reversible one-electron oxidation and reduction and thereby modulates its activity (43). SoxR is maintained *in vivo* in a reduced form, and it is activated by a shift to its oxidized form (17). In *E. coli*, SoxR is oxidized upon exposure to redox-cycling drugs, and its oxidation activates the transcription of its only known target gene, *soxS*. The resultant SoxS protein is itself a transcriptional activator, which activates transcription of over a hundred genes in the SoxRS regulon (93) (Table 1.2).

A model of SoxRS activation is shown in Fig. 1.7. When stress is removed, the oxidized SoxR returns to and is maintained in its reduced state via reducing systems consisting of *rseC* and *rsxABCDGE* (64). Proteolysis rapidly degrades the extant SoxS protein, ending the response (36). SoxR is found in many bacteria, although in non-enterics SoxS is absent, and SoxR directly binds the promoter region of each of the regulon members.
1.7.2 Is $O_2^-$ the physiological inducer of SoxRS?

SoxRS was initially identified as a response against $O_2^-$. People thought so due to the following three reasons. First, SoxRS is induced upon exposure to redox-cycling drugs. These drugs elevate intracellular $O_2^-$ concentration by orders of magnitude above the usual rate. Second, *sodA*, which encodes the $O_2^-$-scavenging enzyme MnSOD, was found to be induced by these drugs aerobically through SoxRS. Failure to effectively induce *sodA* under anaerobic conditions supported the idea that $O_2^-$ is the direct trigger of SoxRS. Subsequent work revealed that two more proteins regulated by SoxRS had demonstrable antioxidant roles: endonuclease IV, which repairs radical-induced DNA damage, and glucose-6-phosphate dehydrogenase, which is a primary source of intracellular NADPH (35, 110). Lastly, SoxR contains a [2Fe-2S] cluster and is activated when the cluster is oxidized (44). $O_2^-$ is known to be able to oxidize exposed [4Fe-4S] clusters in dehydratases (25, 26). Therefore, $O_2^-$ is potentially capable of oxidizing the [2Fe-2S] cluster in SoxR. These data naturally suggested that $O_2^-$ was the trigger of SoxRS response.

However, it has been reported that in *E. coli*, the redox-cycling drug paraquat (PQ) can partially activate the SoxRS regulon even under anaerobic conditions, where $O_2^-$ cannot be produced. The $e^-$ sinks, such as NO$_3^-$ plus PQ, are sufficient to produce MnSOD anaerobically, and $O_2^-$ is not necessary (94). Amy Gort from our lab showed that in *E. coli*, *soxS* is only marginally induced in SOD mutants, which lack both cytosolic superoxide dismutases and accumulate toxic levels of $O_2^-$; and *soxS* induction was not inhibited by extra SOD synthesis (32). These observations imply that $O_2^-$ might not be the direct inducer of SoxRS, and SoxR may sense signals other than $O_2^-$, provided by these redox drugs.
If we take a look at the SoxRS-regulated genes in *E. coli*, many of them do little to detoxify O$_2$ (Table 1.2). Instead, they appear to be focused on reducing the intracellular levels of redox-cycling drugs. For example, the *acrAB* operon encodes a drug efflux system; *micF* encodes an antisense RNA that represses synthesis of the OmpF outer membrane porin; *waaYZ* encodes the LPS modification function that apparently reduces the permeability of the cell envelope (75); *nfsA* encodes nitroreductase A which minimizes the redox cycling attendants upon the univalent reduction of quinones (77); *ygfZ* encodes a plumbagin-modifying enzyme (76); and *nfnB* also encodes a drug modifier (95). In contrast, the OxyR response, which has been identified as the primary H$_2$O$_2$ defensive system in *E. coli*, activated genes that deliberately protect against H$_2$O$_2$. OxyR induces the expression of at least 20 genes (122). Their gene products work cooperatively to scavenge H$_2$O$_2$, and to limit and repair the damages caused by it (Table 1.1). For instance, the peroxidase encoded by *ahpCF* and catalase encoded by *katG* are H$_2$O$_2$-scavenging enzymes; Fur represses iron uptake and limits the amount of iron inside cells; Dps (DNA-binding protein from starved cells) sequesters and prevents iron participation in Fenton reaction (92); YaaA has been suggested to limit the amount of free iron available for Fenton reaction during H$_2$O$_2$ stress (78); MntH, a manganese transporter, imports manganese which has been suggested to replace iron in some metalloenzymes and thereby make them resistant to H$_2$O$_2$ (2, 102); and the Fe-S cluster assembly system Suf is induced during H$_2$O$_2$ stress to replace the H$_2$O$_2$–poisoned Isc system (56).

In *Pseudomonas aeruginosa*, SoxRS can be induced under anoxic conditions (16); and *soxR* mutants show no decrease in O$_2^-$ resistance (91). SoxRs from both *Pseudomonas putida* and *P. aeruginosa* do not control any genes typically involved in
O₂⁻ defence (16, 63, 91). Therefore, SoxRS does not appear to be a O₂⁻ defense in these organisms.

All of the above leaves us with a puzzle: what does SoxRS respond to physiologically?

1.8 Scope of this thesis

1.8.1 Non-redox mononuclear iron enzymes are a new type of cellular target of superoxide.

Since its discovery in 1969, superoxide (O₂⁻) has been recognized as a primary source of oxidative stress. *E. coli* SOD⁻ mutants which lack the ability to scavenge O₂⁻ cannot grow aerobically without supplementation of branched-chain, aromatic and sulfurous amino acids. In the last 40 years, the only cellular target of O₂⁻ that has been identified is a subfamily of [4Fe-4S] dehydratases. There are two such dehydratases in the branched-chain biosynthetic pathways, and their inactivation has been demonstrated to be the origin of branched-chain auxotrophy in the SOD⁻ mutants. However, there are no such dehydratases in the aromatic or sulfurous biosynthetic pathways, implying the existence of other unknown cellular targets. It has been shown that *E. coli* Hpx⁻ cells which lack the ability to scavenge H₂O₂ exhibit aromatic amino acid auxotrophy, as is seen in the SOD⁻ mutants. Therefore, H₂O₂ and O₂⁻ may share the same targets in the aromatic biosynthetic pathways. Previous work done in our lab has demonstrated that non-redox mononuclear iron enzymes are inactivated by H₂O₂ both *in vivo* and *in vitro*. And it has been suggested that the first enzyme in the aromatic biosynthetic pathways – DAHP synthase – is such a non-redox mononuclear iron enzyme. This piece of work is
to investigate whether non-redox mononuclear iron enzymes are cellular targets of $O_2^-$; and if they are, what is the molecular mechanism of $O_2^-$ inactivation of these enzymes.

1.8.2. The SoxRS response is directly activated by redox-cycling drugs rather than by superoxide.

$O_2^-$-sensitive enzymes are present throughout metabolism. Therefore, a number of bacteria and plants exploit this vulnerability: they excrete antimicrobial compounds known as redox-cycling drugs to elevate the production of $O_2^-$ in their competitors. When *E. coli* is exposed to these compounds, its SoxR transcription factor is activated by oxidation of its [2Fe-2S] cluster. $O_2^-$ was initially thought to be the direct activator of SoxR. However, several observations have arisen that do not fit this notion – 1) paraquat could induce *26oda* in *E. coli* even anaerobically if nitrate was supplied; 2) SoxRS is only marginally induced in SOD$^-$ mutants, in which sufficient $O_2^-$ has been accumulated to disable several metabolic pathways; 3) SoxRS activation by paraquat could not be suppressed by SOD overproduction. These observations reopen the question: What is the physiological activator of SoxRS? This part of work was designed to answer the question.
### Table 1.1 Selected OxyR-regulated activities (47).

<table>
<thead>
<tr>
<th>Role</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H₂O₂ scavenging</strong></td>
<td><em>ahpCF</em> (<em>peroxidase</em>)</td>
</tr>
<tr>
<td></td>
<td><em>katG</em> (<em>Catalase</em>)</td>
</tr>
<tr>
<td><strong>Heme synthesis</strong></td>
<td><em>hemH</em> (<em>Ferrochetalase</em>)</td>
</tr>
<tr>
<td><strong>Fe-S cluster assembly</strong></td>
<td><em>sufABCDE</em></td>
</tr>
<tr>
<td><strong>Iron scavenging</strong></td>
<td><em>dps</em></td>
</tr>
<tr>
<td><strong>Iron homeostasis</strong></td>
<td><em>fur</em> (<em>ferric</em> uptake regulator)</td>
</tr>
<tr>
<td><strong>Manganese import</strong></td>
<td><em>mntH</em></td>
</tr>
<tr>
<td><strong>Disulfide reduction</strong></td>
<td><em>trxC</em> (<em>Thioredoxin C</em>)</td>
</tr>
<tr>
<td></td>
<td><em>grxA</em> (<em>Glutaredoxin A</em>)</td>
</tr>
<tr>
<td></td>
<td><em>gor</em> (<em>Glutathione reductase</em>)</td>
</tr>
</tbody>
</table>
Table 1.2 Selected SoxRS-regulated activities (47).

<table>
<thead>
<tr>
<th>Role</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>O$_2$ scavenging</td>
<td>sodA (<em>Mn</em>-containing superoxide dismutase)</td>
</tr>
<tr>
<td>oxidant-resistant</td>
<td>fumC (fumarase C)</td>
</tr>
<tr>
<td>dehydratase isozymes</td>
<td>acnA (aconitase A)</td>
</tr>
<tr>
<td>Fe-S cluster repair</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>yggX</em></td>
</tr>
<tr>
<td></td>
<td>zwf (Glucose-6-phosphate dehydrogenase)</td>
</tr>
<tr>
<td>drug efflux and modification</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>acrAB</em> (Drug efflux pump)</td>
</tr>
<tr>
<td></td>
<td><em>micF</em> (<em>antisense RNA of the outer membrane porine OmpF</em>)</td>
</tr>
<tr>
<td></td>
<td><em>ygfZ</em> (plumbagin modification)</td>
</tr>
<tr>
<td></td>
<td><em>nfsA</em> (drug modification)</td>
</tr>
<tr>
<td></td>
<td><em>nfnB</em> (drug modification)</td>
</tr>
<tr>
<td>iron homeostasis</td>
<td><em>Fur</em> (<em>ferric</em> uptake regulator)</td>
</tr>
<tr>
<td>DNA repair</td>
<td><em>nfo</em> (Endonuclease IV)</td>
</tr>
</tbody>
</table>
Fig. 1.1. Endogenous formation of $O_2^\cdot$. 
Fig. 1.2. Redox-cycling drugs mediate $O_2^-$ production inside cells.
Fig. 1.3. The 4Fe-4S cluster dehydratases are inactivated by $O_2^\cdot$. 
Fig. 1.4. Fur senses intracellular free iron levels and regulates gene expression accordingly.

Feuptake genes
e.g. *iuc*, *feo*, ......
Fig. 1.5. Crystal structure of *E. coli* peptide defomylase (5).
Fig. 1.6. Structures of redox-cycling drugs. Left, paraquat (PQ, methyl viologen); middle, menadione (MD); right, phenazine methosulfate (PMS).
Fig. 1.7. The SoxRS response.

[Diagram of the SoxRS response showing reduced and oxidized SoxR forms and the associated gene expressions.]
1.11 References


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CHAPTER 2: NON-REDOX MONONUCLEAR IRON ENZYMES ARE A NEW TYPE OF CELLULAR TARGET OF SUPEROXIDE

2.1 Introduction

In 1969, McCord and Fridovich identified the existence of a superoxide-specific scavenging enzyme within bovine erythrocytes, which provided the first piece of evidence that superoxide (O$_2^-$) is produced within cells (33). This O$_2^-$–specific enzyme was named superoxide dismutase (SOD). Later, SODs were found in many other organisms (4, 17, 30). Their apparent absence in obligate anaerobes suggests that scavenging O$_2^-$ is critical to survival in aerobic environments. Basal levels of SODs are enough to keep the steady-state concentration of O$_2^-$ at a non-toxic level – 10^{-10} M (14).

In cells lacking SODs (denoted SOD$^-$ mutants), O$_2^-$ concentration may be increased by up to 1000-fold (21).

To date, the only cellular target of O$_2^-$ that has been identified is a subfamily of [4Fe-4S] cluster dehydratases (11-13, 27). In these dehydratases, three of the four iron atoms are coordinated to the sulfur atoms of the cysteine residues in the protein polypeptide, and the forth iron atom is exposed to solvent. This exposed iron is where catalysis takes place: it helps with substrate binding by switching from the tetrahedral to the octahedral conformation; it also provides a local positive charge to drive the dehydration reaction (28). Flint and coworkers found that these solvent-exposed [4Fe-4S] clusters were highly sensitive to O$_2^-$, and the rates of inactivation were measured to be at 10^6 - 10^7 M^{-1}s^{-1} (11). The proposed inactivation mechanism is that O$_2^-$ oxidizes the solvent-exposed [4Fe-4S]$^{2+}$ cluster (Reaction 1), resulting in release of free iron and an inactive [3Fe-4S]$^+$ cluster (Reaction 2).
In fact, inactivation of two such dehydratases, dihydroxyacid dehydratase and isopropylmalate isomerase, results in the branched-chain amino acid auxotrophy in SOD\textsuperscript{-} mutants; and damage to the dehydratases aconitase and fumarase prevents these mutants to catabolize carbon sources that are normally assimilated by the TCA cycle.

However, fundamental aspects of O\textsubscript{2} stress have not been fully understood. SOD\textsuperscript{-} mutants have been shown to contain higher levels of intracellular free iron and thereby suffer more severe DNA damage (25). It was initially proposed that excess amounts of free iron in these mutants resulted from iron leakage from the dehydratases. However, the iron levels were still high even when SOD\textsuperscript{-} mutants were cultured in media in which cells did not rely on the function of these dehydratases. This suggested the existence of other cellular targets from which O\textsubscript{2} can leach iron.

Iron can participate in a wide variety of biochemical reactions inside cells, including redox and non-redox based ones. In redox based reactions, iron changes its redox states during catalysis. Examples of such enzymes include: iron-sulfur cluster enzymes, such as fumarate reductase, in which the clusters form an electron delivery chain between redox partners that are physically separated (20); heme-based enzymes, such as catalase (5); and redox mononuclear iron enzymes, such as iron-containing superoxide dismutase (41). The iron centers are buried in those iron-sulfur cluster enzymes and heme-based enzymes, which make them unlikely to be targeted by O\textsubscript{2}\. In non-redox based reactions, iron does not go through a change in its oxidation states. One such example is the [4Fe-4S] cluster dehydratases, in which the catalytic [4Fe-4S]
cluster is solvent-exposed and is vulnerable to $O_2^-$ oxidation (20). Besides, there is a family of enzymes known as non-redox mononuclear iron enzymes, which utilize a single ferrous iron as the solvent-exposed co-factor. The overall role of iron in these enzymes is similar to the one in dehydratases – to help with substrate binding and to provide a local positive charge. The goal of this study is to deduce if the non-redox mononuclear iron enzymes form a new type of cellular target for $O_2^-$.  

2.2 Results

2.2.1 Endogenous superoxide disrupts Fur metallation.

SOD$^-$ mutants of *Escherichia coli* lack both cytosolic superoxide dismutases (SODs) and therefore accumulate toxic amounts of superoxide ($O_2^-$) inside cells in aerobic environments. In minimal glucose medium, we observed that the iron-import genes, such as *fhuA*, were repressed in the wild type strain but fully expressed in the SOD$^-$ strain (Fig. 2.1A). The gene product of *fhuA* is involved in the transport of siderophore-iron complexes across the outer membrane into the periplasm (31). Like other iron-import genes, the expression of *fhuA* is directly repressed by the Fur:Fe$^{2+}$ complex. Fur stands for ferric uptake regulator, and it senses the intracellular free iron concentration and regulates gene expression accordingly (16). When *E. coli* is replete with iron, Fur binds Fe$^{2+}$ and acquires the activity as a repressor of multiple iron-import genes, including *fhuA*; whereas when *E. coli* starves for iron, Fur is demetallated and the iron import systems are activated to satisfy the organism’s demands for iron. Therefore, the expression levels of iron-import genes reflect the metallation status of Fur. In the wild type strain, *fhuA::lacZ* expression was repressed (Fig. 2.1A), indicating that Fur was
metallated with Fe$^{2+}$. However, the fusion was fully expressed in the SOD$^-$ strain (Fig. 2.1A), which suggested that O$_2^-$ disrupted the metallation of Fur and thereby its ability to properly regulate those genes. One explanation could be that there was not enough iron to metallate Fur in the SOD$^-$ strain. However, our EPR data showed that the SOD$^-$ strain had a higher intracellular free iron level than did the wild type strain (Fig. 2.1B). Therefore, an alternative hypothesis was that O$_2^-$ disrupted Fur metallation. Although O$_2^-$ is largely regarded as a reductant of Fe(III), it does oxidize Fe(II) in some cases, such as in the iron-containing superoxide dismutase. Since Fur acquires the ability as a repressor only when it is bound by Fe(II), it is plausible that O$_2^-$ oxidizes Fur-bound Fe(II) to its ferric form, which then dissociates from the metal-binding site of Fur and leaves it as an apo-protein.

The inactivation of Fur provided us insight into the ability of O$_2^-$ to inactivate other iron-utilizing proteins. Recently, it was reported that several mononuclear iron enzymes are inactivated by hydrogen peroxide (H$_2$O$_2$) (2, 40). These enzymes employ a ferrous iron atom as the catalytic cofactor. Since this iron atom is solvent exposed, small oxidants may have the chance to oxidize it and thereby inactivate the enzyme. We decided to examine whether O$_2^-$ inactivates this type of enzymes in *E. coli*.

### 2.2.2 Mononuclear iron enzymes are damaged in the SOD-deficient strains.

We selected threonine dehydrogenase (Tdh), ribulose-5-phosphate epimerase (Rpe), and peptide deformylase (Pdf) for our study. These three enzymes catalyze different categories of chemical reactions and are involved in diverse cellular pathways. Tdh is involved in threonine catabolism, Rpe functions in the pentose-phosphate
pathway, and PDF releases the formyl group from terminal methionine residue in most nascent proteins. *In vivo*, they have been identified to employ iron as the cofactor, which helps to bind substrate and stabilize anionic reaction intermediates. *In vitro*, they were shown to be able to use some other divalent metals, such as manganese and zinc, although the enzymes became less efficient when they were charged with metals other than iron. We assayed these three enzymes in extracts prepared from aerobic cultures of wild type and SOD− mutant cells. All three enzymes showed substantially lower activities in the SOD− strain than in the wild type (Fig. 2.2). In fact, an SOD− *edd* mutant grew poorly aerobically when gluconate was supplied as the carbon source (Fig. 2.2). The absence of *edd*, the gene encoding 6-phosphogluconate dehydratase, requires gluconate flux through the pentose-phosphate pathway. Inactivation of this pathway in the SOD-deficient strain may result from damage of Rpe.

### 2.2.3 Superoxide rapidly inactivates mononuclear iron enzymes *in vitro.*

To determine whether O$_2^-$ can directly inactivate these mononuclear iron enzymes, the purified enzymes were metallated with Fe$^{2+}$ and then challenged with O$_2^-$ generated *in vitro*. As is shown in Fig. 2.4, all three iron enzymes rapidly lost activity upon exposure to O$_2^-$. The addition of SOD prior to O$_2^-$ challenge completely protected Tdh and Rpe, which confirmed that O$_2^-$ directly inactivated these two enzymes. In the case of Pdf, SOD only partially protected the enzyme, indicating that there were other components reacting with Pdf. O$_2^-$ is generated through the catalysis of xanthine oxidase (reaction 1): molecular oxygen oxidizes xanthine, with itself being reduced to H$_2$O$_2$ and O$_2^-$. Aside from O$_2^-$, two other oxidants in the reaction system – H$_2$O$_2$ and O$_2$ – are also likely to react with Pdf. Since catalase was included in all assays to scavenge
H$_2$O$_2$, the oxidant which might react with Pdf was O$_2$. In fact, we found that O$_2$
inactivated Pdf at a slower rate than did O$_2^-$ (Fig. 2.4C), and that the rates at which Pdf
lost activity correlated with the amounts of O$_2$ present in the assay (Fig. 2.5). To avoid
the complication from O$_2$, our subsequent studies would focus on Tdh and Rpe.

The rate constants with which O$_2^-$ reacts with Tdh and Rpe were also determined.
O$_2^-$ is highly unstable and there is not a good way to measure its concentration.
Therefore, we employed a competition assay system to indirectly measure these
inactivation rates (see Materials and Methods). The measured rates are listed in Table
2.1. Notably, the rates are quite comparable to those at which O$_2^-$ reacts with [4Fe-4S]
cluster dehydratases (11-13, 27). This further convinced us that the inaction of
mononuclear iron enzymes, which we identified as the second type of cellular target of
O$_2^-$, may contribute to the SOD$^-$ phenotypes. In fact, the SOD$^-$ mutant exhibits higher
mutation rates, due to its elevated intracellular iron level. Previously, it was suspected
that the increased iron pool was largely resulted from the damage of those
dehydratases. However, under conditions where most of the known dehydratases were
not expressed, the intracellular iron pool was still high in the SOD$^-$ mutant. We
confirmed this was not due to Fur demetallation, since the SOD$^-$fur$^-$ strain had even
higher unincorporated iron level (Fig. 2.1B). Therefore, the leaching of iron from those
mononuclear iron enzymes during O$_2^-$ stress might contribute to the increase in
intracellular free iron. Indeed, when grown in aerobic environments, the SOD$^-$ strain
overproducing Rpe had even higher free iron level than did its SOD$^-$ parent (Fig. 2.1B).
2.2.4 *In vivo*, superoxide damages mononuclear iron enzymes by enabling mismetallation.

It was initially a puzzle to us how $O_2^-$ could damage those mononuclear iron enzymes *in vivo*: unlike $H_2O_2$, which can form a ferryl radical with the catalytic iron and covalently damage the polypeptide, $O_2^-$ is unlikely to do so. In fact, Tdh employ a cysteine residue to help coordinate the iron atom at its active site (10). This cysteine is known to be oxidatively damaged by $H_2O_2$, because both metals and reductants were required to fully restore its activity after $H_2O_2$ challenge (2). In contrast, $O_2^-$-damaged Tdh completely regained its activity when only $Fe^{2+}$ was added (Fig. 2.6). This suggested that $O_2^-$ damages these enzymes through mechanisms that differ from that of $H_2O_2$.

When we grew wild type and SOD$^-$ mutant anaerobically, stopped *de novo* protein synthesis, and shifted the cultures to aerobic environments, we could watch the loss of enzyme activity over time during aeration. As is shown in Fig. 2.7A, Tdh progressively lost its activity in the SOD$^-$ mutant. After 2 hours of shift to aerobic conditions, Tdh lost ~80% of activity, and longer incubation did not decrease activity any further. The inactivation process was slow *in vivo*, given the fact that Tdh lost >90% activity within 3 min upon $O_2^-$-treatment *in vitro* (Fig. 2.3B).

The loss of Tdh activity could be explained in any of three, non-exclusive ways: 1) the enzyme might exist in the un-metallated apo-form; 2) the protein polypeptide might be degraded; 3) the enzyme might be mischarged with metals other than iron. We tested these three possibilities in turn.
The first possibility could be easily tested by adding metals to the crude extracts of O$_2^-$-stressed cells. The addition of metals did not result in an increase in Tdh activity (Fig. 2.7B), indicating that there was no apo-Tdh accumulated in the O$_2^-$-stressed cells.

To test the second possibility, the crude extracts were incubated with EDTA, a metal chelator, so that whatever metals occupying Tdh active site would be removed. When excess iron was then added to the extracts of O$_2^-$-stressed cells, Tdh activity rebounded to the wild type level (Fig. 2.7C). This suggested that the Tdh polypeptide chain was not degraded in the SOD$^-$ strain.

*In vitro*, Tdh is known to be capable of using various metals for catalysis, including Fe$^{2+}$, Mn$^{2+}$, Zn$^{2+}$ and Co$^{2+}$ (2). *E. coli* typically does not use cobalt, and it lacks a dedicated cobalt transport system. Among the other three physiologically relevant metals, Fe$^{2+}$ provides Tdh the highest $k_{cat}$, which is nearly 5-fold higher than that provided by Mn$^{2+}$ or Zn$^{2+}$. Purified Fe$^{2+}$-metallated Tdh is sensitive to H$_2$O$_2$, whereas the Mn$^{2+}$- and Zn$^{2+}$-metallated enzymes are resistant. Tdh prepared from wild type strain was sensitive to H$_2$O$_2$ (Fig. 2.7D), confirming that this enzyme uses iron under normal conditions. However, Tdh prepared from O$_2^-$-stressed cells was resistant to H$_2$O$_2$ (Fig. 2.7D), implying that it was charged with metals other than iron. We may not examine the metal identity by purifying Tdh from these cells for Inductive Coupled Plasma (ICP) measurements, since metals would exchange during preparation. Instead, we took advantage of their different $K_M$ values. The $K_M$ values of Fe$^{2+}$-, Mn$^{2+}$- and Zn$^{2+}$-metallated Tdh are 6.5 mM, 120 mM, and 2.8 mM, respectively. Tdh from wild type extracts had a $K_M$ consistent with that of the Fe$^{2+}$-metallated enzyme; while Tdh from the SOD$^-$ extracts exhibited a $K_M$ perfectly matching that of the Zn$^{2+}$-metallated enzyme (Table 2.2) (see Materials and Methods for details). Therefore, we concluded that Tdh is
mismetallated with zinc in the $O_2^-$-stressed cells. Similarly, we found that Rpe was also mismetallated with zinc in the SOD$^-$ strain grown aerobically.

### 2.2.5 Mononuclear iron enzymes restore activities in vivo after superoxide stress is removed.

*E. coli* is known to be capable of repairing the oxidatively damaged [4Fe-4S] cluster dehydratases (9), although the molecular mechanism of cluster repair remains unclear. Then an interesting question is whether *E. coli* has also acquired the ability to reactivate those mismetallated mononuclear iron enzymes when $O_2^-$ stress is removed.

We grew the SOD$^-$ strain anaerobically to log phase. Under this condition, Rpe was metallated with Fe$^{2+}$, and its activity was set as 100% (Fig. 2.8A). Then *de novo* protein synthesis was stopped, and aeration was started. After 3 hr of aeration, Rpe lost ~95% of its activity (Fig. 2.8A). The residual activity was resistant to H$_2$O$_2$-treatment and exhibited a $K_M$ value that is characteristic of Zn$^{2+}$-metallated Rpe. Then the cells were spun down, resuspended in anaerobic medium, and incubated anaerobically at 37 ºC. In the absence of new protein synthesis, Rpe fully restored its activity within 60 min of return to anaerobiosis, and it was found to be metallated with Fe$^{2+}$ again (Fig. 2.8A). Similarly, Tdh completely restored its activity within 45 min of return to the anaerobic environments (Fig. 2.8B). It is interesting to note that the time frames for the repair of mononuclear iron enzymes and [4Fe-4S] cluster dehydratases are in the same order of magnitude.

The half-life of zinc dissociation from Rpe is over 8 hr (40). However, Rpe successfully complete its shift from Zn$^{2+}$- to Fe$^{2+}$-metallated form within only 60 min of
return to anaerobiosis. This implied that there existed cellular components which assisted in pulling zinc out of the enzyme. We showed that the compound penicillamine is capable of chelating zinc from Rpe active site, whereas other commonly used metal chelator, such as EDTA, are not able to stip zinc of the enzyme. The structure of penicillamine is shown in Fig. 2.9A, which differs from cysteine in just two methyl groups (-CH₃). Both compounds contain a sulfhydryl (–SH) and an amino (–NH₂) group, which are well known for their metal binding capacity. The intracellular concentration of cysteine has been estimated to be ~200-300 µM. In fact, that amount of cysteine was sufficient to chelate zinc from purified Rpe within an hour (Fig. 2.9B). In contrast, glutathione, which was used as a control due to its well established metal binding ability in vivo, failed to strip zinc of the enzyme at its physiological concentration (5 mM) (Fig. 2.9B). Therefore, we suggest that cysteine is a possible candidate which may help to pull zinc out of those mismetallated mononuclear enzymes and thereby facilitate their repair in vivo.

In summary, we discovered a new type of cellular target of O₂⁻ in E. coli – the nonredox mononuclear iron enzymes. O₂⁻ inactivates this type of enzymes by oxidizing the catalytic ferrous iron and prompting its dissociation from the enzyme active sites. Our model for their inactivation in vivo is shown in Fig. 2.10. Under normal unstressed conditions, the enzyme is metallated with Fe²⁺. In the presence of O₂⁻ stress, iron is oxidized to its ferric form, which dissociates from the enzyme metal-binding site. The inactive apo-enzyme can be repaired by retrieving a ferrous iron atom from the intracellular free iron pool. At the same time, zinc, which is relatively abundant in E. coli, may catch the chance to compete for the metal-binding site. Zn²⁺ binds much more
tightly to the enzyme than does Fe$^{2+}$. Therefore, it traps the enzyme in a mismetallated form, which is much less efficient in catalysis.

2.2.6 In the absence of O$_2^{-}$, overload of zinc poisons TDH by facilitating mismetallation of the enzyme in vivo.

We have shown that, the mononuclear iron enzymes, which normally employ iron as the solvent-exposed cofactor, are mismetallated with zinc in the O$_2^{-}$-stressed cells. In E. coli, zinc is the most abundant soft metal, which is functionally defined by the high affinities for protein sulphydryl groups. In fact, Tdh and Rpe were originally copurified with zinc and were tentatively regarded as zinc enzymes. The “stickiness” of zinc explains its enormously slow dissociation from the metal-binding sites of those enzymes. For example, the half time of metal dissociation from Rpe is over 8 hours for zinc, whereas it is only 50 min for iron (40). Therefore, we may anticipate that, by competing for the metal-binding sites, excess zinc would possibly poison those enzymes even in the absence of oxidative stress.

The intracellular concentration of zinc is mainly held in check by the Zn(II) efflux pump ZntA (39). When the wild type and the zntA-deficient strains were grown in medium without supplementation of extra zinc, Tdh in both strains was metallated with its native metal iron (Fig. 2.11). However, when 150 µM ZnCl$_2$ was added to the growth medium, Tdh in the wild type strain was still metallated with iron, while Tdh in the zntA-deficient strain was mischarged with zinc (Fig. 2.11).

It has recently been reported that zinc, as well as several other soft metals, attack the exposed [4Fe-4S] clusters of dehydratases (43). Here we reported a second type of
cellular target of zinc – the nonredox mononuclear iron enzymes. Both types of enzymes contain key sulphydryl moieties: in the dehydratases, three of the four iron atoms are ligand to the sulfur atoms of cysteine; in the mononuclear iron enzymes, cysteine is located at the metal-binding site to help coordinate the catalytic metal ion. Therefore, our discovery may shed light on the study of toxicity of other divalent soft metals, such as mercury.

2.2.7 Inactivation of DAHP synthase partially results in the aromatic amino acid auxotrophy in SOD⁻ mutants.

SOD⁻ mutants have been shown to be auxotrophs of aromatic amino acids (7). The three aromatic amino acids – Phenylalanine, tyrosine, and tryptophan – are synthesized through the shikimate pathway (19). The first enzyme in the pathway, 3-Deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) Synthase, is predicted to be a mononuclear iron enzyme (32, 38). We decided to test if this enzyme was inactivated by $O_2^-$. Anaerobically, the SOD⁻ mutant grew fine in the absence of aromatic amino acids. Under aerobic conditions, it initially grew fine, then the growth slowed down and eventually stopped (Fig. 2.12A). We picked three time points, which represented different stages of cell growth, and harvested cells for the measurement of DAHP synthase activity. In cells grown anaerobically, DAHP synthase activity stayed high (Fig. 2.12B). In cells cultured aerobically, the enzyme activity decreased over time, and the drop of activity correlated with the decrease in growth rates (Fig. 2.12B). This suggested that damage of DAHP synthase might explain the failure of SOD⁻ mutant to grow aerobically without supplement of aromatic amino acids. To further verify this idea, we
artificially induced the expression of DAHP synthase from a plasmid (pAroG). We found that elevated level of DAHP synthase was able to partially suppress the aromatic auxotrophy in the SOD\textsuperscript{-} mutant (Fig. 2.12C). Therefore, DAHP synthase is a contributor to the aromatic auxotrophy, but there are also other compounds in the aromatic biosynthetic pathway which contribute to this growth defect.

2.3 Experimental procedures

2.3.1 Chemicals and strains.

L-amino acids, Hy-Case Amino, ascorbic acid, antibiotics, α-nitrophenyl-β-d-galactopyranoside, copper-zinc superoxide dismutase (from bovine erythrocytes), catalase (from bovine liver), zinc(II) chloride, diethylenetriaminepentaacetic acid (DTPA), EDTA, NADH, NAD\textsuperscript{+}, tris (2-carboxyethyl) phosphine (TCEP), transketolase (from baker yeast), thiamine pyrophosphate, α-glycerophosphate dehydrogenase-triosephosphate isomerase (from rabbit muscle), ferrous ammonium sulfate hexahydrate, manganese (II) chloride tetrahydrate, D-ribose 5-phosphate disodium salt, D-ribulose 5-phosphate disodiumsalt, and 30% H\textsubscript{2}O\textsubscript{2} were from Sigma. Glycylglycine was from Acros Organics, formate dehydrogenase (Candida boidinii) was from Roche Applied Science, formyl-Met-Ala-Ser was from Bachem, and Tris base was from Fisher.

Strains and plasmids used in this study are listed in Table 2.3. The zntA null mutant was obtained from E. coli Genetic Stock Center. This mutation was introduced into new strains by P1 transduction with selection for linked kanamycin resistance markers (35). The presence of mutations was then confirmed by PCR analysis. The plasmids pCKR101, pAroG, pwks30, and pRpe were introduced into new strains by
electroporation. The single-copy lacZ transcriptional fusion to the fhuA promoter region was integrated into the λ attachment site, while the wild-type genes remained at their native positions (15). The promoter region was amplified using the forward primer 5′-ATATGCCTGCAGCAACAGCAACCTGCTC-3′ and the reverse primer 5′-TATACCGGTACCCATTGGTATATCTCTCTG-3′, which were designed with PstI and EcoR1 restriction sites. The plasmid pAH125 was modified by replacing the kanamycin-resistance cassette with a chloramphenicol-cassette flanked by FLP sites, to permit antibiotic selection under anaerobic conditions. The promoter region was inserted into pSJ501, and the resulting plasmid was confirmed by restriction analysis and sequencing.

2.3.2 Bacterial growth.

LB medium contained 10 g tryptone, 10 g NaCl, and 5 g yeast extract per liter. Defined medium consisted of minimal A or M9 salts supplemented with 0.2% glucose, 5 mg/mL thiamine and 1 mM MgSO₄. Where indicated, Hy-Case Amino was added to a final concentration of 0.2%, and L-amino acids were added to a final concentration of 0.5 mM.

Anoxic growth was performed in a Coy anaerobic chamber under an atmosphere of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. Media and plates used in anaerobic experiments were moved into the chamber while still hot and were allowed to equilibrate with the anaerobic atmosphere for at least 24 h before use. Overnight cultures were diluted to approximately 0.01 OD₆₀₀ and grown for four generations. These exponentially growing cells were then subcultured to an OD₆₀₀ of 0.005–0.01 for subsequent experiments. Anaerobic cultures were grown in a 37°C incubator in the
chamber, and aerobic cultures were grown with vigorous shaking at 37°C. Cell growth was monitored at 600 nm.

2.3.3 EPR spectroscopy.

Intracellular unincorporated iron concentrations were determined following the EPR protocol described previously by Woodmansee and Imlay (42), with minor modifications. Overnight bacterial cultures were prepared anaerobically in M9 media at 37°C. Each overnight culture was then diluted into 1 L of fresh M9 media and aerated rigorously at 37°C. Cultures were grown to an OD_{600} of 0.25. For cells carrying the plasmid prpe, 0.5 mM IPTG was added to the culture at 0.1 OD_{600}, and cells were grown up to 0.25 OD_{600}. Cells were harvested by centrifugation at 8000 r.p.m. for 5 min, and cell pellets were resuspended in 8 ml of M9 medium containing 10 mM DTPA and 20 mM desferrioxamine. The mixture was then incubated at 37°C for 15 min. After the incubation, cells were washed twice with 20 mM cold Tris-Cl (pH 7.4) buffer and then resuspended in cold 20 mM Tris-Cl buffer containing 15% glycerol. ~300 µL of the cell suspension was transferred into an EPR tube and frozen on dry ice. Cell density of the remaining suspension was also measured for final normalization purposes.

EPR spectra were obtained under the following conditions: microwave power, 10 mW; microwave frequency, 9.05 GHz; modulation amplitude, 12.5 Gauss at 100 KHz; time constant, 0.032; and sample temperature, 15 K. Iron concentrations were determined based on iron standards. Iron standards were prepared by making serial dilutions of FeCl₃ in 20 mM Tris-Cl containing 10% glycerol and 1 mM desferrioxamine. Intracellular iron concentrations were calculated by normalizing the iron measurements.
to intracellular volume, using the conversion that 1 ml of 1 OD bacteria collectively contains 0.52 μl of cytosol (21).

2.3.4 Enzyme assays.

Cell extracts were prepared by either sonication or passage through a French press. Total protein content was determined using the Coomassie Blue dye-binding assay (Pierce). β–galactosidase activity was assayed as described (35).

Assays of threonine dehydrogenase (Tdh), peptide deformylase (Pdf), and ribulose 5-phosphate epimerase (Rpe) were conducted anaerobically in the Coy Chamber. Tdh and Pdf were assayed following standard methods (6, 29), with slight modifications. A typical Tdh assay (500 μl) consisted of 50 mM Tris-HCl buffer (pH 8.4), 1 mM NAD+, 30 mM threonine, and the enzyme (pure or from cell extracts). A typical Pdf assay (500 μl) contained 50 mM HEPES buffer with 25 mM NaCl (pH 7.5), 10 mM NAD+, 1 unit of formate dehydrogenase, 1 mM formyl-Met-Ala-Ser, and the enzyme (pure or from cell extracts). Tdh and Pdf activities were monitored by absorbance change at 340 nm. Rpe was assayed (26) by diluting crude extracts or purified enzyme into a 500 μL reaction mixture containing: 50 mM glycyglycine buffer (pH 8.5), 5 mM DTPA, 1 unit of α-glycerophosphate dehydrogenase, 10 units of triosephosphate isomerase, 1 mM ribose 5-phosphate, 1 mM ribulose 5-phosphate, 0.2 mM NADH, and 1 unit of transketolase. Transketolase was obtained in its apo-form and was incubated with 0.2 mM MgCl₂ and 2 mM thiamine pyrophosphate for over 15 min on ice before addition to the assay mixture. NADH consumption was monitored by decrease in A₃₄₀.
2.3.5 Superoxide challenge *in vitro*.

O$_2^-$ was generated in vitro through the catalysis of xanthine oxidase. In the reaction, O$_2$ oxidizes xanthine, with itself being reduced to O$_2^-$ and H$_2$O$_2$. To monitor the loss of enzyme activities upon O$_2^-$ challenge, purified enzymes or cell crude extracts were prepared and mixed with other assay components anaerobically, and catalase was added to prevent interference from H$_2$O$_2$. Then xanthine and xanthine oxidase were added to the mixture aerobically, and enzyme activities were monitored on a spectrophotometer. To ensure efficient O$_2^-$ production, the liquid was pipetted up and down a few times along the cuvette sides. To test the protection effects of SOD on these enzymes, 500 U/ml of SOD was added anaerobically before exposure of the reaction mixture to O$_2^-$ challenge.

2.3.6 Metallation and demetallation of enzymes.

To chelate metals from the active site of Tdh, purified Tdh protein or cell extracts were incubated with 2.5 mM EDTA for 10 min at room temperature anaerobically. In the case of Rpe, 5 mM penicillamine was used instead of EDTA, and the mixture was incubated at room temperature for 40 min anaerobically.

To remetallate the enzymes, Fe(NH$_4$)$_2$(SO$_4$)$_2$ or ZnCl$_2$ was added to a final concentration that was 500 µM extra of the chelator, and the mixture was incubated anaerobically for over 5 min at room temperature.
2.3.7 Metal content determination.

We were able to determine which metal occupies the active site of a mononuclear enzyme, due to the different $K_M$ values corresponding to different metalloforms of the enzyme. For example, the $K_M$ values of Fe$^{2+}$-, Mn$^{2+}$– and Zn$^{2+}$–metallated Tdh are 6.5 mM, 120 mM, and 2.8 mM, respectively (2). Instead of directly measuring the $K_M$'s of Tdh prepared from cells, we may simply measure the ratio of reaction rates at two selected substrate concentrations. The ratios are characteristic for each metalloform of the enzyme. For instance, with 2 mM and 150 mM of threonine in the assay mixture, the ratio is 0.42 for Zn-Tdh, 0.03 for Mn-Tdh, and 0.25 for Fe-Tdh. We can deduce the metal content of Tdh prepared from cells by comparing the ratios to the above standards.

2.4 Discussion

2.4.1 What does $O_2^-$ damage inside cells?

In 1987, the first type of cellular target of $O_2^-$, a subfamily of [4Fe-4S] cluster dehydratases, was identified. The inactivation of two dehydratases in this family results in an almost instant cease of cell growth, when branched-chain amino acids are not supplemented in the growth media. Twenty-five years later, we reported the discovery of a second type of target – the nonredox mononuclear iron enzymes. Although the rate constants with which $O_2^-$ inactivates these two types of targets are quite comparable, the impact of the damage on the mononuclear iron enzymes shows up much more slowly – the SOD' edd' mutant stopped growing several hours after exposure to $O_2^-$ challenge. This slow onset of growth defect matches the progressive diversion of those enzymes from their native metal iron to zinc.
In principle, $O_2^-$ may act either as a reductant or as an oxidant. So far, however, $O_2^-$ has never been shown to damage any biomolecules by reduction. Instead, it oxidizes cellular components and inactivates them. The negative charge of $O_2^-$ largely restricts its ability to oxidize anionic biomolecules, such as sulphydryl groups. In contrast, iron cation centers are excellent $O_2^-$ targets. In fact, $O_2^-$ reacts with both the [4Fe-4S] cluster in dehydratases and the catalytic iron in mononuclear enzymes at quite high rates: $10^6 \text{ M}^{-1}\text{s}^{-1}$ (11). The similarity in rate constants with both types of iron centers makes perfect sense, given that the chemistry in both cases is the oxidation of the exposed iron atom.

Unlike dehydratases, which are solely involved in dehydration reactions, mononuclear iron enzymes catalyze a vast variety of chemical reactions and have larger impact on metabolism. The size of this enzyme family is not yet clear. Iron was not commonly considered to be the native metal that binds to nonredox mononuclear enzymes in vivo. Instead, these enzymes were typically referred as being charged by other divalent cations, such as zinc and manganese. However, the possibility that such enzymes actually use iron in vivo is likely to be overlooked due to ferrous oxidation in experiments conducted in aerobic buffers. Several enzymes that were initially thought to use other metals have been recently demonstrated to employ iron in vivo (8, 37). Cellular conditions are reducing and thus iron exists predominantly in the ferrous form inside cells. Therefore, among the over one hundred nonredox mononuclear enzymes that can be activated in vitro by various transition metals, many of them might actually utilize iron in vivo and thereby are targeted by $O_2^-$. 
2.4.2 Why does *E. coli* employ oxidant-vulnerable mononuclear iron enzymes?

Although iron is good at catalysis due to its chemical flexibility, there are apparent risks of utilizing iron in the aerobic world – reactive oxygen species which are generated as natural byproducts of aerobic metabolism may oxidize the catalytic iron and inactivate the enzyme. This may partially explain the cellular demand for enormously high titers of SODs and catalases to keep the steady-state concentrations of $\text{H}_2\text{O}_2$ and $\text{O}_2^-$ at non-toxic levels.

However, despite of the threat from oxidative damage, *E. coli* retains these iron enzymes rather than employing enzymes specifically utilizing other metals, such as zinc or manganese. This might be explained by the cellular availability and the chemical properties of these metals. Manganese is typically scarce in *E. coli* (3), and it does not bind to protein polypeptide very well. Zinc is relatively abundant, and it binds to proteins very tightly, as is indicated by the fact that a number of proteins utilizing iron *in vivo* were purified with zinc bound at their active sites. However, zinc does not easily shift between tetrahedral and octahedral geometry, which is required by those mononuclear enzymes for catalysis. Therefore zinc is much less efficient in catalyzing the reactions than is iron. These properties of manganese and zinc restrict their usage in the nonredox mononuclear enzymes. In contrast, *E. coli* contains 20-50 µM of intracellular unincorporated iron, which exists almost entirely in the ferrous form (42). Such a ferrous iron pool is likely to be sufficient to activate any enzyme with a high affinity mononuclear site. Besides, iron shifts smoothly between tetrahedral and octahedral geometry, which makes it an excellent cofactor in those enzymes.
2.4.3 How does \textit{E. coli} make sure that mononuclear iron enzymes get the right metal?

Life evolved in an anaerobic environment, when ferrous iron was abundant and zinc was mostly precipitated with sulfide. As the environment became aerobic, O$_2$ oxidized Fe(II) in the environment and made it less available to cells; on the other hand, zinc was released from the precipitants, and zinc concentration increased dramatically. This created a threat to those mononuclear iron enzymes – they might get mismetallated with zinc. In response, bacteria have evolved dedicated mechanisms to tightly control intracellular concentrations of iron and zinc (34, 36, 39). For example, the intracellular zinc level is held in check by the efflux pump ZntA (39).

How metals are properly trafficked to their designated enzymes is basically unknown. It has been suggested that MntS may facilitate manganese delivery to proteins, but its molecular mechanism is not yet understood. There is no evidence for the existence of iron or zinc chaperones. In fact, we showed that Tdh was mismetallated with zinc when cells were overloaded with zinc, which argues against the existence of a special chaperone that helps to initially locate iron to the enzyme and filters out other metals. Besides, when these iron-metallated enzymes were damaged under O$_2$ stress, they eventually ended up with zinc bound. This suggested that there might not be an iron chaperone that puts iron back under stress conditions. Therefore, metallation of those mononuclear enzymes seems to be an open competition.
2.4.4 The differences between the injuries caused by $\text{H}_2\text{O}_2$ and $\text{O}_2^-$.

$\text{H}_2\text{O}_2$ and $\text{O}_2^-$ are two primary sources of oxidative stress. Both oxidants can be generated endogenously through accidentally electron leakage from redox enzymes to molecular $\text{O}_2$. $\text{H}_2\text{O}_2$ can cross cell membranes, therefore exogenous $\text{H}_2\text{O}_2$ can penetrate cells. $\text{O}_2^-$ does not cross membranes, but redox-cycling drugs secreted by certain plants and bacteria can dramatically elevate intracellular $\text{O}_2^-$ production (18). Both $\text{H}_2\text{O}_2$ and $\text{O}_2^-$ can damage [4Fe-4S] cluster dehydratases and mononuclear iron enzymes, by oxidizing the solvent-exposed catalytic iron (2, 11-13, 27, 40). However, $\text{H}_2\text{O}_2$ also covalently damages the protein polypeptides of mononuclear iron enzymes (2), while $\text{O}_2^-$ does not. Therefore, during $\text{H}_2\text{O}_2$ stress, $\text{E. coli}$ induces Dps, an iron-sequestering protein (1), to bind up iron dissociating from those enzymes and prevent it from participating in Fenton reaction (2). In addition, the manganese importer MntH is also induced in $\text{H}_2\text{O}_2$-stressed cells (23). The imported manganese may displace iron in those mononuclear enzymes, and the resultant manganese-loaded enzymes are resistant to $\text{H}_2\text{O}_2$ (2). $\text{O}_2^-$ does not damage protein polypeptides, which may explain the absence of Dps and MntH induction in $\text{O}_2^-$-stressed cells. What needed in $\text{O}_2^-$-stressed cells is the ability to replace mischarged zinc with iron in those mononuclear enzymes. Our work has suggested that cysteine might chelate zinc from those enzymes and thereby facilitate their repair in vivo.
2.5 Tables

Table 2.1 The rate constants with which $O_2^-$ reacts with its selected target enzymes.

<table>
<thead>
<tr>
<th>target enzyme</th>
<th>Inactivation rate constant ($M^{-1} s^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mononuclear iron enzymes</td>
<td></td>
</tr>
<tr>
<td>Tdh</td>
<td>$1.6 \times 10^6$</td>
</tr>
<tr>
<td>Rpe</td>
<td>$8.2 \times 10^5$</td>
</tr>
<tr>
<td>[4Fe-4S] dehydratases</td>
<td>$(2-6) \times 10^6$</td>
</tr>
</tbody>
</table>

The inactivation rate constants for mononuclear iron enzymes were measured in this study (see Experimental procedures); those for [4Fe-4S] cluster dehydratases were cited from (11-13, 27).
Table 2.2 Tdh is mismetallated with zinc in O$_2$-stressed cells.

<table>
<thead>
<tr>
<th>Tdh sample</th>
<th>V(2 mM) / V(150 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tdh from cell extracts</td>
<td></td>
</tr>
<tr>
<td>wt</td>
<td>0.24</td>
</tr>
<tr>
<td>SOD−</td>
<td>0.43</td>
</tr>
<tr>
<td>Purified Tdh</td>
<td></td>
</tr>
<tr>
<td>Fe-Tdh</td>
<td>0.25</td>
</tr>
<tr>
<td>Mn-Tdh</td>
<td>0.03</td>
</tr>
<tr>
<td>Zn-Tdh</td>
<td>0.42</td>
</tr>
</tbody>
</table>

The theoretical $K_M$ values for Zn-, Mn-, and Fe-metallated Tdh are 2.8 mM, 120 mM, and 6.5 mM, respectively (2). According to Michaelis-Menten kinetics, reaction rates depend on substrate concentrations and $K_M$ values: \( v = \frac{v_{max} \text{ [threonine]}}{K_M + \text{[threonine]}} \). Cell extracts were prepared from aerobically cultured wild type and SOD-deficient strains, the reaction rates at 2 mM and 150 mM threonine concentrations were measured, and the ratios were calculated.
<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB1157</td>
<td>$F^{-}$ thr-1 leuB6 proA2 his-4 thi-1 argE2 lacY1 galK2 rpsL supE44 ara-14 xyl-15 mtl-1 tsx-33</td>
<td>(22)</td>
</tr>
<tr>
<td>PN134</td>
<td>Same as AB1157 plus (sodA:: Mud PR13)25 (sodB-kan) 1-Δ2</td>
<td>Lab collection</td>
</tr>
<tr>
<td>KK210</td>
<td>Same as AB1157 plus fur::Tn5 zbf-507::Tn10</td>
<td>(24)</td>
</tr>
<tr>
<td>KK216</td>
<td>Same as PN134 plus fur::Tn5 zbf-507::Tn10</td>
<td>(24)</td>
</tr>
<tr>
<td>AB1157/pCKR101</td>
<td>Same as AB1157 plus pCKR101</td>
<td>This study</td>
</tr>
<tr>
<td>AB1157/pAroG</td>
<td>Same as AB1157 plus pAroG</td>
<td>This study</td>
</tr>
<tr>
<td>PN134 / pCKR101</td>
<td>Same as PN134 plus pCKR101</td>
<td>This study</td>
</tr>
<tr>
<td>PN134 / pAroG</td>
<td>Same as PN134 plus pAroG</td>
<td>This study</td>
</tr>
<tr>
<td>GS45</td>
<td>Same as AB1157 plus $\lambda$(fhuA::lacZ)</td>
<td>This study</td>
</tr>
<tr>
<td>GS46</td>
<td>Same as PN134 plus $\lambda$(fhuA::lacZ)</td>
<td>This study</td>
</tr>
<tr>
<td>AB1157/pWKS30</td>
<td>Same as AB1157 plus pWKS30</td>
<td>This study</td>
</tr>
<tr>
<td>AB1157/pRpe</td>
<td>Same as AB1157 plus pRpe</td>
<td>This study</td>
</tr>
<tr>
<td>PN134/ pWKS30</td>
<td>Same as PN134 plus pWKS30</td>
<td>This study</td>
</tr>
<tr>
<td>PN134/pRpe</td>
<td>Same as PN134 plus pRpe</td>
<td>This study</td>
</tr>
<tr>
<td>GS73</td>
<td>Same as AB1157 plus zntA::kan</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmids</td>
<td>Description</td>
<td>Developer</td>
</tr>
<tr>
<td>------------------------</td>
<td>------------------------------------------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>pCKR101 pBR328</td>
<td>derivative containing $\text{lac}^R$</td>
<td>Jeff Gardner</td>
</tr>
<tr>
<td>pAroG</td>
<td>pCKR101 derivative containing $\text{aroG}$</td>
<td>Jason Sobota</td>
</tr>
<tr>
<td>pWKS30</td>
<td>a low-copy-number vector carrying the ampicillin-resistance marker</td>
<td>James Slauch</td>
</tr>
<tr>
<td>pRpe</td>
<td>pWKS30 derivative containing $\text{rpe}$</td>
<td>Jason Sobota</td>
</tr>
</tbody>
</table>
2.6 Figures

Fig. 2.1. (A) O$_2^-$ disrupts Fur mettallation in M9 medium. The wild type (wt) and SOD-deficient (SOD-) strains were grown aerobically in glucose/amino acid M9 medium to an OD$_{600}$ of ~0.25. Where indicated, 1 mM dipyridyl was added, and cells were aerated for an additional 30 min. (B) Intracellular free iron levels. Cells were cultured as described in (A). To induce Rpe expression, 0.5 mM IPTG was added to the culture at ~0.1 OD$_{600}$. EPR samples were prepared as described in “SI Materials and Methods”. Wild type, AB1157; SOD-, PN134; fur-, KK210; and SOD- fur-, KK216.
Fig. 2.2. Mononuclear iron enzymes are damaged in the SOD-deficient strains. The wild type (wt) and SOD-deficient (SOD⁻) strains were grown aerobically in glucose/amino acid minimal A medium to an OD₆₀₀ of ~0.3, and enzyme activities were measured.
Fig. 2.3. Pentose phosphate pathway fails in SOD' mutants. Cells were precultured anaerobically and then diluted into aerobic gluconate/aminoc acid minimal A medium. Cell growth was monitored thereafter.
Fig. 2.4. Superoxide rapidly inactivates mononuclear iron enzymes in vitro. Purified Tdh (A), Rpe (B) and PDF (C) were metallated with Fe(II) anaerobically. $O_2^-$ was generated in vitro aerobically by xanthine and xanthine oxidase. Where indicated, 500 U/ml of SOD was added before exposure to $O_2^-$. 
Fig. 2.5. \( \text{O}_2 \) inactivates Fe-bound peptide deformylase. Purified peptide deformylase (PDF) was loaded with Fe(II) anaerobically. Where indicated, aerobic buffer was added to the anaerobic assays in the anaerobic chamber. To reach the saturated concentration, \( \text{O}_2 \) was introduced by pipetting along the sides of the cuvette aerobically. Catalase and SOD were included in all assays.
Fig. 2.6. Unlike H$_2$O$_2$, O$_2^-$ did not oxidize the Cys residue at the active site of Fe-Tdh. Purified Tdh was metallated with Fe(II) anaerobically. (A) Fe-Tdh was treated with 50 µM H$_2$O$_2$ for 3 min anaerobically, and catalase was then added to scavenge residual H$_2$O$_2$. (B) Fe-Tdh was treated with O$_2$ for 3 min in the presence of catalase, and 500 U/ml of SOD was then added to scavenge residual O$_2^-$. Where indicated, Fe(NH$_4$)$_2$SO$_4$ (0.5 mM) and TCEP (0.5 mM) were added to reactivate the enzyme.
Fig. 2.7. (A) Tdh progressively loses activity in the SOD\(^-\) strain. Wild type (wt) and SOD\(^-\) mutant were grown anaerobically to an OD\(_{600}\) of 0.2. \textit{de novo} protein synthesis was stopped, and cultures were shifted to aerobic conditions. At indicated time points, aliquots were taken to measure Tdh activity. (B) Tdh does not accumulate in the apoprotein form in O\(_2\)\(\)-stressed cells. Cell extracts were prepared from SOD\(^-\) strain which had been aerated for 120 min. Where indicated, 0.5 mM of Fe(NH\(_4\))\(_2\)(SO\(_4\))\(_2\) was added to the extracts. (C) Tdh protein polypeptide is not degraded in O\(_2\)\(\)-stressed cells. Cell extracts were prepared from wild type and SOD\(^-\) strain which had been aerated for 120 min. The extracts were incubated anaerobically with 2.5 mM EDTA at RT for 10 min. Then Fe(NH\(_4\))\(_2\)(SO\(_4\))\(_2\) was added anaerobically to a final concentration that was 0.5 mM higher than EDTA. (D) Tdh from O\(_2\)\(\)-stressed cells is not metallated with iron. The cell extracts from part (C) were treated with 50 \(\mu\)M H\(_2\)O\(_2\) anaerobically.
Fig. 2.8. Rpe (A) and Tdh (B) restore activity in vivo after superoxide stress is removed. The SOD deficient strain (SOD−) was grown anaerobically at 37 ºC to an OD600 of ~0.20. Then de novo protein synthesis was stopped, and cells were shifted to aerobic conditions. After 3 hours of aeration at 37 ºC, cells were returned to anaerobic conditions. At the subsequent time points, aliquots were harvested for measurement of Rpe and Tdh activity.
Fig. 2.9. (A) Chemical structures of penicillamine, cysteine, and glutathione. (B) Physiologically relevant concentration of cysteine was able to efficiently chelate zinc from Rpe in cell extracts. The SOD deficient strain (SOD−) was grown anaerobically at 37 °C to an OD600 of ~0.25. Then de novo protein synthesis was stopped, and cells were aerated at 37 °C for 3 hours. Penicillamine, cysteine or glutathione was added to the crude extracts at indicated concentrations. After one hour incubation at RT, Rpe activity was measured.
Fig. 2.10. Model for \textit{in vivo} inactivation of mononuclear iron enzymes by $O_2^\cdot$. 
Fig. 2.11. Even in the absence of $\text{O}_2^-$ stress, excess intracellular zinc causes Tdh mismetallation *in vivo*. The wild type strain (wt) and the zinc efflux deficient mutant ($\Delta zntA$) were grown in glucose/amino acid medium in the absence (A) or presence (B) of 150 $\mu$M ZnCl$_2$. To test the $\text{H}_2\text{O}_2$-sensitivity of Tdh, 50 $\mu$M $\text{H}_2\text{O}_2$ was incubated with the crude extracts at RT for 3 min. To chelate metals from Tdh active site, the crude extracts were anaerobically incubated with 2.5 mM EDTA at RT for 10 min. Fe(NH$_4$)$_2$(SO$_4$)$_2$ or ZnCl$_2$ was then added anaerobically to a final concentration that was 0.5 mM higher than EDTA.
Fig. 2.12. Inactivation of DAHP synthase in SOD\textsuperscript{+} mutants partially resulted in aromatic amino acid auxotrophy. (A) The SOD\textsuperscript{+} mutant grew poorly aerobically in the absence of aromatic amino acids. SOD\textsuperscript{+} cells were pre-cultured anaerobically in minimal glucose medium without aromatic amino acid supplementation. They were then diluted into the same medium both anaerobically and aerobically. Cell growth was monitored thereafter. (B) The drop in DAHP synthase activity correlated with the decrease in growth rate. Cells were harvested at the indicated time points for the measurement of DAHP synthase activities. (C) Artificial induction of DAHP synthase from a plasmid partially relived the growth defect. Strains containing the empty vector or the DAHP synthase-overproducing plasmid (pAroG) were pre-cultured anaerobically with tyrosine present. They were then diluted into the same medium aerobically. 0.1 mM IPTG was included in both precultures and cultures to induce the plasmid.
2.7 References


41. Vance CK and Miller AF (2001) Novel insights into the basis for *Escherichia coli* superoxide dismutase’s metal ion specificity from Mn-substituted FeSOD and its very high E(m). *Biochemistry* 40(43):13079-13087.


CHAPTER 3: THE SOXRS RESPONSE OF *ESCHERICHIA COLI* IS DIRECTLY ACTIVATED BY REDOX-CYCLING DRUGS RATHER THAN BY SUPEROXIDE

3.1 Introduction

Superoxide dismutase (SOD) was discovered accidentally (42), and for a period its physiological function was controversial. However, Hassan and Fridovich determined that the *E. coli* manganese SOD isozyme was strongly induced when cells were exposed to redox-cycling drugs in aerobic medium, a combination that generates superoxide inside cells (21). Subsequently, the Demple and Weiss labs found that this regulation is mediated by a transcription factor with an iron-sulfur cluster that is oxidized during drug treatment (18, 23, 55). Superoxide was known to rapidly oxidize the catalytic iron-sulfur clusters of dehydratases (11-13, 30), and so it seemed likely that superoxide itself was the signal that activated SoxR and thereby induced SOD. The regulator was therefore named SoxR, for superoxide response regulator (18, 55).

SoxR is a dimeric transcription factor, with each monomer containing a [2Fe-2S] cluster (22). When aerobic *E. coli* is exposed to redox-cycling drugs such as paraquat or menadione, the cluster undergoes a reversible one-electron oxidation and gains the ability to activate the transcription of *soxS*, the gene immediately adjacent (9, 10, 14, 15). The resulting SoxS protein is itself a transcription factor that activates the expression of more than one hundred genes in the SoxRS regulon (49). When oxidative stress abates, the oxidized SoxR is returned to its reduced state via reducing systems encoded by *rseC* and *rsxABCDGE* (35). Proteolysis rapidly degrades the extant SoxS...
protein, ending the response (19). SoxR is found in many bacteria, although in non-enterics SoxS is absent and SoxR directly binds the promoter region of each of the regulon members (6).

What environmental circumstances might create superoxide stress inside bacteria? Superoxide is continuously generated by the adventitious oxidation of redox enzymes (25), but studies with *E. coli* indicate that the basal amount of SOD is enough to keep its steady-state concentration at non-toxic levels. Superoxide does not cross membranes, so exogenous superoxide cannot penetrate cells (29). To date, then, the only situation that elevates intracellular superoxide to levels that warrant SOD induction are the conditions that were originally explored by Hassan and Fridovich—the presence of redox-cycling drugs (21) (The structures of three classes of redox-cycling drugs are shown in Fig. 1). It is now recognized that these compounds are released by both plants and bacteria as devices to inhibit the growth of competitors (27, 46). For example, plumbagin, a naphthoquinone, was originally isolated from the plant *Plumbago* (57); juglone, another quinone, occurs naturally in the Juglandaceae family and is recognizable as the yellow residue on the leaves and seeds of the black walnut (32). Both compounds are effective herbicides that allow the parent plant to dominate a habitat. Phenazines are commonly excreted by bacteria, including Pseudomonads, Streptomyces, and *Pantoea agglomerans* (56). They exert toxic effects on other bacteria. In addition, man-made viologens such as paraquat (PQ, methyl viologen) are also used as herbicides. Each of these drugs can penetrate into the cell interior, where they abstract single electrons from the reduced flavins or metal centers of redox enzymes. The reduced drug can then transfer the electron to oxygen, generating
superoxide (20). This redox-cycling behavior can elevate intracellular superoxide formation by orders of magnitude above the usual rate.

Thus the induction of SOD by the SoxRS regulon provides a critical defense against these drugs. Other components of the regulon focus on limiting the intracellular levels of these drugs. For example, the acrAB encodes a drug efflux system (37). The micF gene encodes an antisense RNA that represses synthesis of the OmpF outer membrane porin (1), while waaYZ encodes an LPS modification function (34); together, the induction of these genes apparently reduces the permeability of the cell envelope so that redox drugs cannot easily enter. Other induced proteins—NfsA, YgfZ, and NfnB—detoxify redox drugs by modifying them (36, 52). The presence of these genes in the SoxRS regulon confirms that this system exists to defend the cell from such redox-cycling drugs.

However, several observations have arisen that do not fit the notion that SoxR responds to superoxide per se. First, workers found that paraquat could induce sodA in *E. coli* even in anaerobic habitats, if nitrate was supplied (51). Under those conditions superoxide could not be present. Further, experiments showed that SoxRS is poorly induced in SOD− mutants of *E. coli*, despite the accumulation of sufficient superoxide to disable several key metabolic pathways. The same study showed that SoxRS activation by paraquat could not be suppressed by SOD overproduction; together these results indicated that superoxide was neither sufficient nor necessary for SoxRS activation $\text{.}$ Both groups suggested alternative mechanisms, including the possibilities that redox drugs might either directly oxidize SoxR or that they might deplete NADPH and thereby slow the SoxR reducing system. More recently, studies in *Pseudomonas aeruginosa* replicated the observation of anaerobic SoxR activation by paraquat/nitrate, although the
authors pointed out the caveat that nitric oxide, which can be formed as a by-product of nitrite reduction, might activate SoxR by degrading its iron-sulfur cluster (7). Finally, the SoxR regulons of both *P. aeruginosa* and *P. putida* do not seem to involve enzymes, such as SOD, that explicitly defend cells against superoxide (7).

These results reopen the question: What is the physiological activator of SoxRS? As a corollary, to what extent might these drugs threaten cells through mechanisms that do not involve superoxide?

### 3.2 Results

**3.2.1 Superoxide is not the physiological activator of SoxRS.**

Hydroperoxidase mutants (*katG katE ahp*; denoted Hpx) cannot scavenge H$_2$O$_2$. However, the toxic impact of H$_2$O$_2$ is abated because OxyR-regulated genes are induced to protect against H$_2$O$_2$ stress. Thus an HpxOXyR strain is extremely sick in aerobic medium (47). SOD mutants (*sodA sodB*) lack both cytosolic superoxide dismutases and therefore accumulate toxic amounts of O$_2^-$. SOD mutants grew poorly in aerobic medium compared to wild type strains (Fig. 3.1 A). We wondered whether the toxic impact of O$_2^-$ would be similarly blunted because SoxRS response was induced. However, the SOD soxS strain was no sicker than the SOD mutant (Fig. 3.1 A). The possible explanations for that could be either SoxRS is not activated in SOD mutants, or the genes SoxRS controls do not protect against O$_2^-$. In order to test the first possibility, we measured the degree of SoxRS induction using strains containing *soxS::*lacZ* fusions. Our lab previously reported that *soxS* is not
significantly activated in SOD mutants (16), and we reproduced the results. soxS was barely induced in SOD mutants, despite the fact that they contained toxic amount of O$_2^-$ to completely inactivate metabolic pathways. In contrast, soxS was highly induced by paraquat (PQ) both in the presence and absence of SODs (Fig. 3.1 B). Therefore, endogenous O$_2^-$ is a poor inducer of SoxRS compared to drugs.

The implication from the above experiment is that SoxR senses some signal provided by drugs other than O$_2^-$. To verify this idea, we examined whether excess SOD could inhibit soxS induction by drugs. There were two strains: one expresses normal amount of FeSOD, the other contains a sodB overproducing plasmid and produces 20-fold higher of FeSOD activity. If O$_2^-$ were the inducer of SoxRS, the half inducing dose would be 20-fold higher in the overproducer. However, we found that there was no difference in the induction patterns between those two strains in response to drugs from all three categories – viologen (PQ), phenazine (PMS) and menadione (quinone) (Fig. 3.1 1C). Therefore, soxS induction by redox-cycling drugs does not require O$_2^-$. 

3.2.2 Most SoxRS-controlled genes do not mitigate superoxide stress.

We have shown that SoxRS is pretty much off in an SOD mutant. Then we wanted to test the second possibility: whether SoxRS, if artificially being induced, could confer protection against O$_2^-$. The soxS gene was overexpressed directly from the tac promoter using psoxS, and enzyme assays confirmed that this construct overexpressed zwf as strongly as when 100 µM paraquat was added, indicating that the SoxRS regulon was fully induced. We observed that, like its SOD^- parent (PN134), the SoxRS-induced strain remained unable to grow in glucose medium unless aromatic, sulfur-containing, and
branched-chain amino acids were supplemented. Even in amino-acid supplemented medium, SoxRS induction did not noticeably improve the growth rate of the SOD\(^{-}\) mutant.

The primary toxic effect of \(O_2\) in *E. coli* is damage of labile 4Fe-4S cluster dehydratases (11-13, 30). The incapacity of growth without branched-chain amino acids has been attributed to oxidative damage of 4Fe-4S cluster dehydratases in the biosynthetic pathways. We measured the impact of soxS overexpression on the activity of one of these enzymes, 6-phosphogluconate dehydratase (Edd). Edd contains a solvent-exposed 4Fe-4S cluster and is expressed when gluconate is provided as the carbon source. soxS overexpression improved Edd activity in SOD mutants, which came from a gene called *yggX* (Fig. 3.2). *yggX*, a member of the SoxRS regulon, encodes an 11-KD cytoplasmic protein and has been proposed to be involved in Fe-S cluster metabolism during \(O_2\)\(^{-}\) stress (17, 50). SOD mutants, when cultured in air, produced only 25% of Edd activity compared to wild type strain. If we overproduced either soxS or *yggX* in an SOD mutant, Edd activities were increased by more than 2.5-fold. However, if we knocked out *yggX*, soxS overproduction could no longer improve Edd activity in an SOD mutant (Fig. 3.2). YggX improved Edd activity by facilitating Fe-S cluster repair during \(O_2\)\(^{-}\) stress (Fig. 3.3).

There are more than a hundred genes in the SoxRS regulon, many of which have unknown functions (2). *sodA*, encoding the Mn-containing superoxide dismutase, apparently defends against \(O_2\)\(^{-}\); *acnA* and *fumC*, encoding oxidant-resistant isozymes, replace \(O_2\)\(^{-}\)-damaged enzymes; and *yggX* facilitates Fe-S cluster repair under stress. Aside from those, SoxRS-controlled genes seem to be focused upon coping with drugs. *acrABC* operon encodes a drug efflux system; *micF* encodes an antisense RNA which
represses transcription of an outer membrane porin called OmpF; waaYZ encodes the LPS modification function and enhances resistance against multiply drugs (34); ygftz encodes a plumbagin-modifying enzyme (35); nfnB also encodes a drug modifier (58). O$_2^-$ protection may be just a minor part of the SoxRS system.

3.2.3 Cellular respiration reoxidizes redox-cycling drugs and enables them to activate SoxR.

Redox-cycling drugs were unable to induce the manganese-containing SOD under anaerobic conditions, which fit the notion that O$_2^-$ was the activator of SoxRS. An alternative explanation, however, might be that only the oxidized drugs generate the SoxR signal; when oxygen is unavailable to reoxidize them, the drugs quickly accumulate in a reduced, inactive form. Indeed, when paraquat is added to anaerobic cultures, both the media and the cells turn the blue color of the reduced paraquat radical species; the color immediately dissipates when air is introduced.

We examined expression of soxS itself under these anaerobic conditions. Unlike sodA, the soxS gene could still be induced, although the induction level was much smaller than when oxygen was present (Fig. 3.4A).

Other workers reported that paraquat can induce sodA in the absence of oxygen if nitrate is present (57). An ambiguity attending these experiments was the fact that nitric oxide, which is formed in trace amounts during denitrification, can itself activate SoxR (8). We found that soxS expression was also induced strongly when nitrate was supplied to the cell (Fig. 3.4A). Further, fumarate, another respiratory substrate, also improved induction (see the insert of Fig. 3.4B). Fumarate, nitrate, and oxygen are
progressively better respiratory substrates for \textit{E. coli}, and so their parallel effects on \textit{soxS} expression suggested that paraquat might be recycled in vivo via oxidation by the electron transport chain. Indeed, the inducing effect of paraquat/fumarate was lost when we eliminated the capacity for fumarate-directed respiration, by deleting the operon encoding fumarate reductase. Deletion of the \textit{nar} genes, which encode the primary nitrate reductase, substantially diminished the inducing effect of nitrate/paraquat, but the treatment could still promote \textit{soxS} expression to some extent (Fig. 3.4 \textit{A}). The residual effect may have been due to the remaining nitrate reductase isozymes or, possibly, to the formation of nitric oxide. Fumarate was unable to stimulate anaerobic \textit{soxS} transcription in mutants that lacked respiratory quinones (\textit{menA ubiA}), but it did so in \textit{ndh nuo} mutants that lack the NADH dehydrogenases through which most of reducing equivalents enter the chain (Fig. 3.4 \textit{B}). These results suggest that drugs accumulate in their reduced (non-inducing) forms unless they can transfer their electrons to an oxidized pool of respiratory quinones.

The ability of the respiratory chain to directly oxidize reduced redox drugs was confirmed in vitro. Inverted respiratory vesicles containing fumarate reductase were prepared from cells grown in anaerobic media with fumarate. Paraquat radical was generated by adding dithionite to paraquat in anaerobic buffer. The paraquat-radical spectrum was quickly bleached upon the addition of both inverted respiratory vesicles and fumarate (Fig. 3.5).

To verify that the role of oxygen in SoxR activation is simply to recycle reduced drugs, we sought to use an alternative oxidant, ferricyanide, in its place. Ferricyanide is a small chemical oxidant which is known to be able to oxidize the quinone pool. In the absence of respiratory substrates, the enhancing effect of aerobic respiration upon \textit{soxS}
expression was replicated by the provision of ferricyanide (Fig. 3.6). In fact, when ferricyanide was used at high concentrations, the anaerobic induction level of soxS was even higher than when oxygen was present.

To verify that anaerobic soxS expression reflects the oxidation of SoxR, this protein was overexpressed in vivo, and EPR spectroscopy was performed to monitor the redox state of its [2Fe-2S] cluster during drug exposure (9, 14). In the absence of redox-cycling drugs, the cluster exhibited an EPR spectrum that is typical of reduced [2Fe-2S]^+ clusters. When paraquat was added to anaerobic cells, we observed a gradual disappearance as the paraquat concentration increased (Fig. 3.7 A), indicating the oxidation of SoxR. Menadione and PMS had the same effect.

The above observations collectively demonstrated that O_2^- is not the signal that SoxR senses. The key role of oxygen is to regenerate the oxidized drugs, which, directly or indirectly, trigger SoxR oxidation.

3.2.4 Drugs directly oxidize SoxR anaerobically.

The redox state of SoxR is determined by the kinetic equilibrium between its reduction and its oxidation; therefore, redox-cycling drugs must either slow the first process or accelerate the second. NAD(P)H is the probable electron source for SoxR reduction (28), and one possibility is that redox-cycling drugs might lower NADPH levels and thereby lead to SoxR activation. To test whether NADPH depletion can have this effect, we examined a zwf pnt mutant, which lacks the two primary mechanisms by which _E. coli_ generates NADPH. In minimal glucose medium this mutant grew with a four-hour doubling time due to its extreme deficiency in NADPH. In the absence of
redox drugs, the strain showed a minimally elevated expression of \textit{soxS}. However, it still responded strongly to PQ (Fig. 3.8 A), suggesting that NADPH depletion itself is a weak trigger of SoxRS induction. The \textit{rsx} and \textit{rseC} gene products constitute the primary reducing systems for SoxR in \textit{E. coli} (28). When we eliminated either or both of these, we again observed slightly higher basal levels of \textit{soxS} induction, but once more the mutants showed typical SoxRS induction patterns in response to PQ (Fig. 3.8 B). Therefore, the activating effect of the drugs is not due to an inhibition of these SoxR-reducing systems.

The obvious alternative is that redox-cycling drugs directly oxidize SoxR. To examine this possibility, we purified SoxR protein from the \textit{soxR}-overproducing strain XA90/pKOXR. SoxR was eluted in an oxidized form, which showed a spectrum typical of \([2\text{Fe-2S}]\) with absorption maxima at 414 and 450 nm (fig. 3.9). It could be reduced under anaerobic conditions by dithionite, generating an EPR signal that is characteristic of reduced \([2\text{Fe-2S}]^+\) clusters. When we treated the reduced SoxR protein with redox drugs in the absence of oxygen, this EPR signal rapidly disappeared (Fig. 3.7 B), corresponding to the oxidation of the SoxR cluster to the \([2\text{Fe-2S}]^{2+}\) form.

These data were consistent with the fact that small-molecule mediators had been used to determine the SoxR reduction potential (10); however, the rates of SoxR oxidation by potential oxidants have not been reported. The oxidized cluster absorbs light at 414 nm, and so we used visible spectroscopy to measure the rates at which redox drugs react with SoxR in the absence of oxygen. The rate constants are listed in Table 3.1. To use these values in calculating the rate at which SoxR might be oxidized by a drug in vivo, it is necessary to know the intracellular concentration of the drug. \textit{E. coli} cells that had been cultured with paraquat were harvested, washed, and lysed, and
the released paraquat was reduced with dithionite and quantified by spectroscopy. We found, for example, that incubation with 200 μM paraquat—an inducing dose—led cells to accumulate at least 700 μM intracellular paraquat. This value is likely to be an underestimate, since some paraquat was lost during the washing process, but it is sufficiently high to predict that intracellular paraquat can oxidize SoxR within the time frame of the biological response (see Discussion).

Oxidation rate constants were also measured when reduced SoxR was exposed to molecular oxygen and to hydrogen peroxide. These values are very low (Table 3.2), and they explain why neither aeration nor physiological (low-micromolar) H₂O₂ stress activates SoxR in vivo (62). Finally, the question of whether O₂⁻ can directly oxidize SoxR (eq. 1) was examined in two ways:

(1) \[ O_2^- + SoxR[2Fe-2S]^+ + 2H^+ \rightarrow H_2O_2 + SoxR[2Fe-2S]^{2+} \]

First, exposure of reduced SoxR to xanthine oxidase, a potent source of superoxide, did not lead to any apparent loss of [2Fe-2S]⁺ absorbance over 5 min. An equivalent exposure inactivated fumarase A by > 90% within 20 s. Second, the presence of reduced SoxR did not detectably diminish the rate at which xanthine-oxidase-generated O₂⁻ transferred electrons to cytochrome c (Fig. 3.10). Given the rate constant with which O₂⁻ reacts with cytochrome c, we deduce that the rate constant with which SoxR degrades O₂⁻ must be lower than 1000 M⁻¹ s⁻¹ (Materials and Methods). This value is far too low for SoxR to respond to physiological levels of O₂⁻ (see Discussion).
3.2.5 Redox-cycling drugs exert toxic effects in the absence of oxygen.

Our data suggest that SoxRS can be fully activated without O\textsubscript{2}. Then are there any benefits to induce SoxRS anaerobically? Redox drugs slowed bacterial growth in anaerobic nitrate media, although higher drug doses were needed than in aerobic media (Fig. 3.11), indicating a defense against drugs is useful under anaerobic conditions. 120 μM PMS almost completely blocked the growth of the wild type strain under anaerobic conditions; upon exposure to 40 μM PMS, cells lagged and then grew, suggesting that adaptation occurred. A lower dose created a branched-chain amino acid auxotrophy; wild-type cells eventually adapted, while a ΔsoxR mutant did not (Fig. 3.12). Branched-chain auxotrophy results from the oxidation of the two [4Fe-4S] cluster dehydratases in that pathway, dihydroxyacid dehydratase and isopropylmalate isomerase (33, 35). Therefore, the implication was that PMS can damage vulnerable dehydratases even in the absence of oxygen, and that cells respond by inducing SoxRS.

The obvious hypothesis was that redox drugs might directly oxidize the clusters of dehydratases, as they do that of SoxR. Indeed, assays confirmed that paraquat, menadione, and PMS treatments each inactivated fumarase B inside anaerobic cells (Fig. 3.13 A). This observation explains why Fnr does not override the induction of *fumC*, which encodes a cluster-free fumarase isozyme. These drugs were also able to directly inactivate purified fumarase A in vitro (Fig. 3.13 B). Fumarase was protected from drugs by its substrate, L-malate (Fig. 3.14 A), indicating that the drugs damaged the enzyme by interacting with its active site; activity was fully restored when the inactivated enzyme was subsequently incubated with ferrous iron, dithiothreitol, IscS, and cysteine, a cocktail that rebuilds damaged iron-sulfur clusters (Fig. 3.14 B). The
anaerobic inactivation constants were measured in vitro to be $10^2$–$10^4$ M$^{-1}$ s$^{-1}$ at zero degrees C.

### 3.3 Experimental procedures

#### 3.3.1 Chemicals and strains.

Fumaric acid, potassium nitrate, and HEPES were obtained from Fisher Scientific. Sodium dithionite was purchased from Fluka. Sodium molybdate was from Mallinckrodt. Methyl viologen (paraquat), phenazine methosulfate, menadione sodium bisulfite, potassium ferricyanide, L-amino acids, isopropyl β-D-1-thiogalactopyranoside (IPTG), o-nitrophenyl-β-D-galactopyranoside (ONPG), xanthine, xanthine oxidase from bovine milk, copper-zinc superoxide dismutase from bovine erythrocytes, horse heart cytochrome c, ferrous ammonium sulfate, dithiothreitol (DTT), L-malic acid, and 30% hydrogen peroxide were from Sigma-Aldrich.

Strains and plasmids used in this study are listed in Table 3.1. Null mutations were created by the Red recombinase method (Datsenko and Wanner, 2000) and were confirmed by PCR analysis. Mutations were introduced into new strains by P1 transduction with selection for linked antibiotic resistance markers (43). The presence of mutations was then confirmed by phenotype, enzyme assay, or PCR analysis. To create plasmids overexpressing *yggX*, the *yggX* ORF was PCR-amplified from *E. coli* GC4468 by using the forward primer 5′- ACTGCGAATTCTAGCGGCTCCGTGGAG and the reverse primer 5′- TCACCTCTAGGGAGATGAGCAACGCG. The EcoRI and XbaI sites are underlined. PCR products were digested and cloned into pCKR101 vector behind a *tac* promoter to generate the *pyggX* plasmid. The insert was verified by
sequencing. The psoxS plasmid was made using the same method. The forward primer was 5′- GTGACGAATTCCGCGGATACCGCCAC and the reverse primer was 5′- TCACCTCTAGAGCGAAGGCAGTGCCGC.

3.3.2 Bacterial growth.

Luria-Bertani medium (LB) contained (per liter) 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl. Defined media contained minimal A salts (43) supplemented with 0.2% glucose, 1 mM MgSO₄ and 5 mg/ml thiamine. Where indicated, L-amino acids were added to a final concentration of 0.5 mM. Nitrate medium contained 20 mM KNO₃ and 5 μM sodium molybdate, and fumarate medium contained 20 mM fumaric acid. The plasmid pCKR101 and its derivatives were maintained with 100 μg/ml ampicillin, and pKK1 was maintained with 12 μg/ml tetracycline.

Anaerobic experiments were conducted in a Coy anaerobic chamber under an atmosphere of 85% N₂/10% H₂/5% CO₂. Media and plates used in anaerobic experiments were moved into the chamber while still hot and were allowed to equilibrate with the anaerobic atmosphere for at least 24 hr prior to use. Chemicals used in anaerobic experiments were brought into the chamber and dissolved in anaerobic buffer. Overnight cultures were diluted to approximately 0.01 OD₆₀₀ and grown for 4 generations. These exponentially growing cells were then subcultured to an OD₆₀₀ of 0.005-0.01 for subsequent experiments. Anaerobic cultures were grown in a 37 °C incubator in the chamber, and aerobic cultures were grown with vigorous shaking at 37 °C.
3.3.3 SoxR purification.

SoxR protein was purified from aerobic cultures of the soxR overexpressing strain XA90/pKOXR (45), which was kindly provided by Dr. Huangen Ding from Louisiana State University. All purification steps were performed at 4 °C under aerobic conditions as described (28). The isolated enzyme exhibited the features of the oxidized cluster (SI Figure 4). It lacked an apparent EPR signal (< 5% of the signal after subsequent dithionite addition), indicating that essentially all of the purified enzyme was isolated in the oxidized form.

3.3.4 Enzyme assays.

Cell extracts were prepared by either sonication or passage through a French press. Total protein content was determined using the Coomassie Blue dye-binding assay (Pierce). β–galactosidase activity was assayed as described (43). The activity of 6-phosphogluconate dehydratase (Edd) was assayed by the two-step procedure for determination of pyruvate (16). Fumarase activity was assayed by monitoring the conversion of 50 mM l-malate to fumarate at an OD of 250 nm in 50 mM sodium phosphate buffer (pH 7.3) (40). Oxidant-resistant fumarase C activity was measured after fumarase A and B activities had been inactivated by 2 mM H$_2$O$_2$ for 10 min at room temperature.

SOD activity was assayed by the xanthine oxidase-cytochrome c method (41). To test whether SoxR could inhibit cytochrome c reduction by competing for O$_2^-$, purified SoxR protein was reduced by dithionite anaerobically, excess dithionite was removed by centrifugation through a spin column (Amicon Ultra 3 K MWCO, Millipore) at 7000 g and
4 °C anaerobically, and reduced SoxR was added to the assays before the addition of xanthine. Xanthine oxidase makes $O_2^-$, and $O_2^-$ reduces cytochrome c. The rate constant for cytochrome c reduction is $2.6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. By comparing the rates of cytochrome c reduction in the presence and absence of reduced SoxR, one can calculate how fast $O_2^-$ is consumed by SoxR. In order to estimate how fast fumarase A reacts with $O_2^-$, purified fumarase A was added to the assays before the addition of xanthine.

3.3.5 Reconstitution of Fe-S clusters.

Damaged clusters in the [3Fe-4S] form were chemically repaired by incubation with 100 µM Fe(NH$_4$)$_2$(SO$_4$)$_2$ and 2.5 mM dithiothreitol (DTT) anaerobically for 8 min at room temperature. Reconstitution of more extensively degraded Fe-S clusters was carried out by incubation with 500 µM Fe(NH$_4$)$_2$(SO$_4$)$_2$, 5 mM DTT, 2.5 mM cysteine, and purified IscS (0.16 mU) in anaerobic buffer at room temperature for 20 min. To purify IscS, the gene iscS was inserted into pET15b, and the resulting pIscS-(His)$_6$ was overexpressed in E. coli BL21 strain. The overexpressed IscS-(His)$_6$ was purified using His Gravitrap (GE Healthcare) (Jang & Imlay, manuscript submitted).

3.3.6 EPR spectroscopy.

The reduced [2Fe-2S]$^+$ cluster has a characteristic EPR spectrum near $g=1.93$; the oxidized cluster is EPR-silent (14). Thus, one can monitor the redox state of SoxR by EPR. Whole-cell EPR samples were prepared from SoxR-overproducing strain XA90/pKOXR grown anaerobically in LB plus ampicillin. To overexpress SoxR protein, IPTG was added when the cells reached $A_{600} = 0.10$, and the cells were incubated at 37
°C for another 2 hr. Then the cells were left untreated or treated with redox-cycling drugs for 40 min anaerobically. The cells were harvested, washed quickly with minimal salts, and resuspended at 1/500th of the original culture volume in cold anaerobic 10% glycerol. The cell suspensions (300 μl) were then transferred into EPR tubes and immediately frozen on dry ice.

For study of purified SoxR, 15 μM protein was reduced by dithionite anaerobically. Excess dithionite was removed as described above. The reduced SoxR protein was then treated with redox-cycling drugs anaerobically, transferred into EPR tubes, and frozen. EPR spectra of [2Fe-2S]^+ clusters were obtained at the following settings: microwave power, 1 milliwatt; microwave frequency, 9.05 GHz; modulation amplitude, 12.5 G at 100 KHz; time constant, 0.032; and sample temperature, 15 K.

3.3.7 Inactivation of purified fumarase A.

Fumarase A was purified from anaerobic cultures of the fumA-overexpressing strain SJ50 (33). All purification steps were conducted anaerobically at chamber temperature (27 °C). Inactivation of fumarase A was accomplished by the addition of redox-cycling drugs to the purified enzyme in anaerobic buffer. Damage was terminated by addition of 20 mM L-malate prior to anaerobic assay.

To inactivate fumarase A by O₂-, 0.01-0.04 μM fumarase A was exposed to 50 μM xanthine and 14 mU/ml xanthine oxidase in aerobic buffer, which generated 0.9 μM O₂· in the first 1 min. At time points, damage was terminated by the addition of > 22 U/ml SOD. L-malate was then added, and fumarase A activity was measured.
3.3.8 Oxidation of purified SoxR.

The SoxR protein was purified in the oxidized form, which displayed characteristic absorption maxima at 414 and 450 nm. When reduced with dithionite, these absorption maxima are diminished (28). Therefore, one can track the redox state of SoxR by monitoring $A_{414}$. Purified SoxR protein was reduced by dithionite anaerobically. Dithionite was removed as described above. Reduced SoxR was then transferred to anaerobic cuvettes and exposed to PQ (100-700 µM), menadione (100-700 µM), PMS (30-300 µM) or $H_2O_2$ (0.2-2 mM) anaerobically. The oxidation of SoxR was monitored by following $A_{414}$. When $O_2$ was the oxidant under investigation, reduced SoxR was diluted into aerobic buffer and $A_{414}$ was monitored. In the case of $O_2$, xanthine and xanthine oxidase were added to SoxR aerobically.

3.3.9 Determination of intracellular PQ concentrations.

Dithionite reduces PQ to a blue radical, which absorbs at 395 nm. The strain TN530 was cultured anaerobically to an OD$_{600}$ of 0.10, and PQ was added to concentrations at which SoxRS was highly induced. After 1 hr incubation at 37 °C, cells were harvested and washed. Crude extracts were made by French press. Dithionite was then added, and PQ concentrations were determined by the absorption at 395 nm ($\varepsilon_{395}=0.0132 \mu M^{-1}$). PQ concentrations in the extracts were then converted to concentrations in the cells based on 1 ml of 1 OD$_{800}$ of cells contains 0.5 µl of cytoplasmic volume (26).
3.3.10 Detection of PQ oxidation by cell membranes.

The wild-type strain (GC4468) was cultured anaerobically in 600 ml of minimal medium supplemented with 0.2% glucose and 40 mM fumarate. At an OD$_{600}$ of 0.4, cells were harvested, washed with 50 mM KPi pH 7.0, and resuspended in the same buffer. Cells were lysed by passage through a French press, and cell debris was removed by centrifugation at 7,000 g x 20 min at 4°C. Inverted vesicles were then isolated by ultracentrifugation (27,000 rpm x 2 hrs at 4°C). Vesicles were resuspended by pipetting in ~2.5 ml of anaerobic 50 mM KPi pH 7.0. To detect PQ oxidation, the absorbance of buffer containing 30 µM dithionite and 20 µL of cell membranes (a total volume of 500 µL) was blanked at 385-600 nm. When 5 µM PQ was added anaerobically to the mixture, an absorption peak at 395 nm was observed, which represented the formation of PQ radical. The signal disappeared when 0.1-5 mM fumarate was added anaerobically. Bleaching was not observed if either fumarate or respiratory vesicles were omitted.

3.4 Discussion

The experiments reported here indicate that the SoxR response comprises a general defense against redox-cycling drugs. The apparent mechanism of SoxR activation is depicted in Figure 9. Redox-cycling drugs directly oxidize SoxR; when respiratory substrates are present, the reduced drugs then transfer electrons to the quinones of the respiratory chain. The latter reaction recycles the drugs, enabling them to continue to oxidize both SoxR and other target enzymes in the cell.
3.4.1 SoxR is configured to be a non-specific sensor of univalent oxidants.

Phenazines, quinones, and viologens are efficient oxidants of exposed flavins and metal centers, since their one-electron reduction does not require the scission or creation of new bonds and therefore does not require facilitation by particular enzymic functional groups. Thus the ability of these drugs to oxidize redox enzymes is determined largely by whether they can get close to their cofactors. The crystal structure of SoxR shows that its [2Fe-2S] sensory cluster is exposed at the surface of the protein (61), which means that redox drugs of a wide variety of shapes and sizes can approach and oxidize it; this protein is therefore ideal as non-selective sensor of univalent oxidants. In contrast, superoxide dismutases have evolved so their redox metals are buried at the bottom of a narrow cleft that excludes molecules larger than diatomic superoxide (54), and in fact they do not react with other molecules. This comparison suggests that the structure of SoxR evolved to sense a variety of oxidants, rather than superoxide.

The oxidizability of SoxR is assisted by the low potential of its [2Fe-2S] cluster (-285 mV), which provides a strong driving force for electron transfer to even moderate oxidants (10). The SoxR cluster also has a key feature that the dehydratase [4Fe-4S] clusters lack: when it is oxidized it does not degrade. Instead the oxidized [2Fe-2S]$^{2+}$ cluster remains intact and is quickly reduced again when oxidants are removed, presumably allowing the regulon to respond quickly to changing circumstances (9). In contrast, the degraded clusters from oxidized [4Fe-4S] dehydratases are rebuilt slowly, particularly in iron-poor environments. In this respect SoxR provides an interesting contrast with the E. coli Fnr transcription factor, whose [4Fe-4S] cluster is destroyed as
the crux of its oxygen-sensing mechanism (33). Perhaps SoxR is calibrated to sense the degree of redox stress, whereas Fnr provides a black-or-white report on oxygenation.

3.4.2 Can SoxR sense superoxide?

The fact that SoxR reacts directly with redox drugs does not, of course, mean that it cannot also react with superoxide. However, in our experiments the rate of its reaction with superoxide fell below the detection limit, indicating that the rate constant was less than 1000 M$^{-1}$ s$^{-1}$. Prior work suggested that in aerobic *E. coli* the cytoplasmic superoxide level is about 0.1 nM, and a ten-fold increase to 1 nM creates enough stress that metabolic pathways begin to fail. At the latter concentration—even employing 1000 M$^{-1}$ s$^{-1}$, the upper limit for SoxR reactivity—one calculates that the half-time for SoxR oxidation would be 8 days. Thus SoxR is not capable of sensing the amount of superoxide that comprises a threat to the organism.

One can also calculate that paraquat, not the superoxide it generates, is the more important oxidant of SoxR in paraquat-stressed cells. Our measurements show that when *E. coli* is treated with 200 µM paraquat the intracellular rate of non-respiratory oxygen consumption (a.k.a., cyanide-resistant respiration) reaches a maximum of ca. 1.1 mM s$^{-1}$; this value indicates the formation of 2.2 mM s$^{-1}$ superoxide, a very high rate. Yet, given that even uninduced cells contain 3600 U/ml SOD, one calculates that the steady-state level of superoxide in these paraquat-challenged cells is only 80 nM. This concentration is high enough to oxidize dehydratases (k = 10$^6$-10$^7$ M$^{-1}$ s$^{-1}$) with a half-time of 10 seconds, but the half-time for the oxidation of SoxR by superoxide (k < 1000 M$^{-1}$ s$^{-1}$) must exceed 2 hours. In contrast, SoxR oxidation by the 700 M intracellular
paraquat that accumulates in this circumstance ($k = 30 \text{ M}^{-1} \text{s}^{-1}$) should exhibit a half-time of only 30 seconds. Thus even in extremely superoxide-stressed cells, superoxide levels are inadequate to activate SoxR—but the drug itself is a sufficient inducer.

One might wonder, then, why SOD mutants exhibit slight SoxRS activation. In these cells the superoxide level may rise far higher than in SOD-proficient cells, even when the latter are treated with redox drugs. A level of up to $5 \text{ M}$ is theoretically possible (26), which would oxidize SoxR with a half-time of about 2 minutes—conceivably fast enough to partially override the reducing activity of the Rse/Rsx systems. However, as the preceding calculations show, these levels of superoxide cannot be achieved in wild-type cells that contain SOD, even with toxic levels of redox drugs. It is also possible that the metabolic dysfunction of SOD mutants diminishes the efficiency of the Rse/Rsx systems.

The sluggish reactivity of the SoxR cluster with superoxide contrasts with the high reactivities of the clusters of dehydratases, which react with superoxide at rates that are at least 1000-fold greater ($10^6$-$10^7 \text{ M}^{-1} \text{s}^{-1}$) (11). Similarly, the rate constant for the oxidation of SoxR by $\text{H}_2\text{O}_2$ ($0.5 \text{ M}^{-1} \text{s}^{-1}$) is far lower than those of dehydratases ($10^3$-$10^4 \text{ M}^{-1} \text{s}^{-1}$) (33). Why the disparity? The key difference may be that the dehydratases do not provide a cysteinyl ligand to the catalytic iron atom of their [4Fe-4S] clusters (32) (39). This arrangement is appropriate for catalysis, since the under-coordinated iron atom is available to directly bind the carboxylate and hydroxyl oxygen atoms of its physiological substrates (32, 39). Thus, when substrate is not bound, the exposed iron atom can directly bind oxidants such as hydrogen peroxide or superoxide in its place. Direct binding is essential for electron transfer to hydrogen peroxide, which is cleaved upon reduction and therefore requires an inner-sphere electron exchange from the metal. While in principle superoxide can receive electrons through an outer-sphere
mechanism, as redox drugs do, electron hopping is disfavored by the requirement that superoxide be protonated to $\mathrm{HO}_2^-$ prior to electron transfer, since $\mathrm{HO}_2^-$ is a plausible product but $\mathrm{O}_2^{2-}$ is not. Protonation likely occurs while superoxide is complexed to the dehydratase cluster.

Unlike dehydratases, SoxR provides four sulfur ligands—two from cysteine residues, and two from inorganic bridging sulfur atoms—to both of the iron atoms of its $[2\text{Fe}-2\text{S}]$ cluster (4, 61). This arrangement may impede direct binding by superoxide and $\mathrm{H}_2\mathrm{O}_2$ and explain its lesser reactivity with them. Consistent with this idea, the reaction rates of SoxR and of dehydratases differ only by about 10-fold when the oxidants are drugs or molecular oxygen, which do not need to bind clusters directly in order to oxidize them.

3.4.3 The threat of redox-active drugs.

The prevalence of redox-cycling drugs in real-world habitats is not clear; to date workers have studied their excretion by only a handful of plants and bacteria. The toxicity of these compounds seems to suggest that organisms excrete these drugs in order to suppress the growth of their competitors. That certainly appears to be the case with plants, such as walnut trees, which denude the ground below them through quinones that are extruded from their dropped leaves. The effect is that walnut seeds can find bare soil. A similar strategy has been attributed to bacteria that secrete such compounds. However, Wang, Newman, and co-workers have recently pointed out that the release of phenazines by biofilm-forming bacteria might have the effect of permitting electron delivery to terminal oxidants, such as iron precipitates, that lie at a distance from the bacterium (59). Alternatively, this process might solubilize the iron, making it
bioavailable (60). Explication of the regulatory signals that control phenazine excretion might illuminate its purpose.

In any case, these drugs are toxic to bystander bacteria. While redox compounds are best known for their ability to generate reactive oxygen compounds (21), we found that their univalent redox activity allows them to damage iron-sulfur clusters directly. Other detrimental effects are also known. By shuttling electrons from NADPH-reduced enzymes to the respiratory chain, they comprise an NADPH sink that might impede reductive processes in the cell. Of course, simply by oxidizing these reductases, the drugs may act as competitive inhibitors, too. Prior work has shown that overoxidation of NADH dehydrogenase II, in fact, converts it to a form that is inactive in vivo (24).

Many of the redox drugs are also effective Michael reagents that undergo addition reactions with cellular nucleophiles—a reaction that does not involve redox cycling at all (3, 44, 53, 58). Through the latter activity they form adducts with available thiols, including those of glutathione and enzyme active-site cysteine residues, and with the exocyclic amino groups of nucleic acids (3, 44). It is not clear which of these mechanisms comprises the greatest threat to a growing cell; presumably the answer varies from compound to compound, since facility at Michael activity and redox-cycling behavior need not correlate. SOD-deficient E. coli are hypersensitive to these drugs in aerobic complex medium, indicating that in the absence of SOD their toxicity is mediated primarily by superoxide (21). However, SOD overproduction does not provide much more resistance to wild-type strains (24), suggesting that in drug-exposed cells the SoxRS-mediated induction of SOD proceeds to a point that something other than superoxide becomes the primary threat. Interestingly, Adriamycin and daunomycin are related quinone-based cancer therapeutics whose use is limited by their toxicity; most
work suggests that Michael adduction, rather than redox-cycling, underlies their ability to kill mammalian cells (5, 31, 38, 58).

Thus it is reasonable that bacteria that are targeted by these compounds use a defensive regulon that does not depend upon superoxide as a signal, that functions even in anaerobic environments, and that addresses injuries that are different than those produced by reactive oxygen species. This perspective—that the threat is the drugs themselves, not just the superoxide they create—may also clarify the physiological logic of the SoxRS regulon. Since NADPH depletion is a likely consequence of redox-cycling, it seems plausible that the SoxRS induces glucose-6-phosphate dehydrogenase and ferredoxin:NADP oxidoreductase in order to restore the NADPH pool. YggX evidently assists the repair of iron-sulfur clusters, whether damage is mediated by superoxide or directly by the drugs themselves. Yet the predominant strategy of the *E. coli* SoxRS regulon is to minimize the intracellular drug concentration through mechanisms that impede their entry, chemically modify them, or pump them out. Drug excretion, in fact, is the SoxR-regulated function that seems most widespread, being found, for example, in Pseudomonad SoxR regulons that do not induce SOD at all (48). Thus the emerging view is that the SoxRS regulon is perhaps best construed as a defensive system against redox-active drugs rather than against superoxide; superoxide is apparently only one element of the threat that these drugs comprise.
### 3.5 Tables

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<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype</th>
<th>Source or reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>AB1157</td>
<td>$F^{+}$ thr-1 leuB6 proA2 his-4 thi-1 argE2 lacY1 galK2 rpsL surE44 ara-14 xyl-15 mtl-1 tsx-33</td>
<td>Lab collection</td>
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<tr>
<td>AS358</td>
<td>Same as TN530 plus $(sodA::Mud PR13)25 (sodB-kan)1-Δ2$</td>
<td>(22)</td>
</tr>
<tr>
<td>AS395</td>
<td>Same as TN530 plus $(sodA::Mud PR13)25$</td>
<td>(22)</td>
</tr>
<tr>
<td>AS396</td>
<td>Same as AS395 plus pKK1</td>
<td>(22)</td>
</tr>
<tr>
<td>BW25113</td>
<td>$lacI$ $rrnB$ $lacZ$ $hsdk$ $araBAD$, $rhaBAD$</td>
<td>Lab collection</td>
</tr>
<tr>
<td>DJ901</td>
<td>Same as GC4468 plus $ΔsoxRS901$</td>
<td>(24)</td>
</tr>
<tr>
<td>GC4468</td>
<td>$Δ$ $lacU169$ rpsL</td>
<td>Lab collection</td>
</tr>
<tr>
<td>GS07</td>
<td>Same as PMTA01 plus psoxS</td>
<td>This study</td>
</tr>
<tr>
<td>GS09</td>
<td>Same as PMTA21 plus pCKR101</td>
<td>This study</td>
</tr>
<tr>
<td>GS10</td>
<td>Same as PMTA21 plus pyggX</td>
<td>This study</td>
</tr>
<tr>
<td>GS11</td>
<td>Same as PMTA21 plus psoxS</td>
<td>This study</td>
</tr>
<tr>
<td>GS63</td>
<td>Same as TN530 plus $rseC::kan$</td>
<td>This study</td>
</tr>
<tr>
<td>GS64</td>
<td>Same as TN530 plus $rsxC::kan$</td>
<td>This study</td>
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<td>GS65</td>
<td>Same as TN530 plus $ΔrsxC$ and $rseC::kan$</td>
<td>This study</td>
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<tr>
<td>GS66</td>
<td>Same as TN530 plus (\Delta cyd \Delta (cyo\text{ABCDE})456::kan)</td>
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<td>GS67</td>
<td>(zej\text{-223}::\text{Tn10 F rpsL gal nuo ndh }\Delta\text{lacZ1} + \lambda (soxS::lacZ }\Delta\text{soxR}))</td>
<td>This study</td>
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<tr>
<td>GS68</td>
<td>(ubiA420\ menA401\ \Delta\text{lacZ1} + \lambda (soxS::lacZ }\Delta\text{soxR}))</td>
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<tr>
<td>GS69</td>
<td>Same as TN530 plus (narG::kan)</td>
<td>This study</td>
</tr>
<tr>
<td>GS70</td>
<td>Same as TN530 plus (\Delta(frd\text{ABCD})8)</td>
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<tr>
<td>PMTA01</td>
<td>Same as PN134 plus (galP::\text{Tn10 and yggX::kan})</td>
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<tr>
<td>PMTA21</td>
<td>Same as PN134 plus (galP::\text{Tn10})</td>
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<tr>
<td>PN134</td>
<td>Same as AB1157 plus ((sodA::\text{Mud PR13})25, (sodB-kan)1-}\Delta2)</td>
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<tr>
<td>PP135</td>
<td>Same as GC4468 plus (yggX::kan)</td>
<td>(63)</td>
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<tr>
<td>RB2</td>
<td>Same as PN134 plus (soxS3::\text{Tn10})</td>
<td>This study</td>
</tr>
<tr>
<td>SJ33</td>
<td>Same as BW25113 plus (\Delta\text{fumCA})</td>
<td>Lab collection</td>
</tr>
<tr>
<td>SJ50</td>
<td>(\Delta(katG17::\text{Tn10})1 (\text{ahpC-ahpF})\text{del kan::'}\text{ahpF} \Delta(katE12::\text{Tn10})1 \Delta\text{fumCA }\Delta\text{fumB::Cm plus pflumA})</td>
<td>Lab collection</td>
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<tr>
<td>SP14</td>
<td>Same as TN530 plus (pnt::\text{Tn5(\text{kan}), }\Delta(\text{edd-zwf})22\ \text{zeb-1}::\text{Tn10})</td>
<td>Lab collection</td>
</tr>
<tr>
<td>TN530</td>
<td>Same as GC4468 plus (\lambda(\text{soxS::lacZ }\Delta\text{soxR}))</td>
<td>(57)</td>
</tr>
<tr>
<td>XA90/pKEN2</td>
<td>(\Delta(\text{lac pro})\text{XIII ara nalA argE thi (F' lac}^{\text{Rd}}\text{ ZY proAB})) plus pKEN2</td>
<td>(57)</td>
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Table 3.1 (continued)

<table>
<thead>
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<th>Strain or plasmid</th>
<th>Relevant genotype</th>
<th>Source or reference</th>
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<tbody>
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<td>XA90/pKOXR</td>
<td>$\Delta(lac\ pro)_{XIII<del>ara</del>nalA<del>argE</del>thi~(F'~lacI^{ql}<del>ZY</del>proAB)}$ plus expression plasmid for $soxR$</td>
<td>(57)</td>
</tr>
<tr>
<td></td>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pCKR101</td>
<td>pBR328 derivative containing $lac^{R}$</td>
<td>Jeff Gardner</td>
</tr>
<tr>
<td>pyggX</td>
<td>pCKR101 derivative containing $yggX$</td>
<td>This study</td>
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<tr>
<td>psoxS</td>
<td>pCKR101 derivative containing $soxS$</td>
<td>This study</td>
</tr>
<tr>
<td>pKEN2</td>
<td>a high copy phagemid vector</td>
<td>(57)</td>
</tr>
<tr>
<td>pKOXR</td>
<td>pKEN2 derivative containing $soxR$ behind a $tac$ promoter</td>
<td>(57)</td>
</tr>
<tr>
<td>pKK1</td>
<td>pBR328 derivative containing $sodB$</td>
<td>Kay Keyer</td>
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Table 3.2 Rate constants for the oxidation of purified SoxR and fumarase A by univalent oxidants.

<table>
<thead>
<tr>
<th>Oxidant</th>
<th>Rate constant for SoxR oxidation ((M^{-1} \text{s}^{-1}, 25 ^\circ \text{C}))</th>
<th>Rate constant for fumarase A oxidation ((M^{-1} \text{s}^{-1}))</th>
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</thead>
<tbody>
<tr>
<td>(\text{O}_2)</td>
<td>0.5 ± 0.3</td>
<td>300 (^{[A]})</td>
</tr>
<tr>
<td>(\text{H}_2\text{O}_2)</td>
<td>0.8 ± 0.3</td>
<td>(10^5^{[B]})</td>
</tr>
<tr>
<td>(\text{O}_2^–)</td>
<td>&lt;1000</td>
<td>(10^8^{[C]})</td>
</tr>
<tr>
<td>PMS</td>
<td>167 ± 5</td>
<td>((2 ± 0.015) \times 10^3^{[D]})</td>
</tr>
<tr>
<td>MD</td>
<td>48 ± 3</td>
<td>(65 ± 4^{[E]})</td>
</tr>
<tr>
<td>PQ</td>
<td>30 ± 3</td>
<td>(3.6 ± 0.05 \times 10^2^{[F]})</td>
</tr>
</tbody>
</table>

PMS, phenazine methosulfate; MD, menadione; PQ, paraquat (methyl viologen). The rate constants of fumarase A oxidation were measured at 25 \(^\circ\)C for \(^{[A]}\) \(\text{O}_2\) (11), \(^{[B]}\) \(\text{H}_2\text{O}_2\) (33), and \(^{[C]}\) \(\text{O}_2^–\) (11); those for \(^{[D]}\) PMS, \(^{[E]}\) MD and \(^{[F]}\) PQ were measured in this study at 0 \(^\circ\)C.
3.6 Figures

Fig. 3.1. The SoxRS response is not activated by superoxide.  (A) Strains AB1157 (wild type) (●), PN134 (sodA sodB) (■) and RB2 (sodA sodB soxS) (▲) were pre-cultured in anaerobic LB medium to log phase. At time zero, the pre-cultures were diluted into aerobic medium, and growth was monitored.  (B) Cultures of SOD-proficient strain (SOD⁺) TN530 (soxS::lacZ) and SOD-deficient strain (SOD⁻) AS358 (sodA sodB soxS::lacZ) were grown in LB medium to an OD₆₀₀ of 0.1. The cultures were each split into two flasks, 160 µM PQ was added to one, and the other was left untreated. All cultures were aerated for an additional 45 min. The error bars represent the range of activity measured for three independent cultures. (C). Strains AS395 (sodA soxS::lacZ) (closed symbols) and AS396 (sodA soxS::lacZ psodB) (open symbols) were grown aerobically in LB medium to an OD₆₀₀ of 0.05. The cultures were diluted into flasks containing PMS (diamonds), MD (triangles) or PQ (squares). The cultures were further incubated at 37°C for 60 min. Solution assays showed that SOD activity was 20-fold higher in strains carrying the psodB plasmid.
Fig. 3.2. The SoxRS response protects a [4Fe–4S] dehydratase by inducing yggX. Anaerobic cultures of SOD-deficient strains growing in LB plus 0.2% gluconate and 100 µg ml⁻¹ ampicillin were diluted into the same aerobic medium to an OD₆₀₀ of 0.05. IPTG was added (100 µM). The cultures were grown at 37°C for ~3 h to an OD₆₀₀ of 0.4, and the activity of 6-phosphogluconate dehydratase (Edd) was determined. GS09, SOD⁺ vector; GS11, SOD⁻ psoxS; GS10, SOD⁻ pyggX; GS07, SOD⁻ yggX psoxS. Edd activity in a wild-type strain was defined as 100%.
Fig. 3.3. YggX accelerates 4Fe-4S cluster repair during O$_2^*$ stress. SOD-deficient strains were grown anaerobically to an OD$_{600}$ of 0.20 in LB medium supplemented with 0.2% gluconate, 100 µg/ml ampicillin and 100 µM IPTG. Cells were shifted to air, aerated for 30 min at 37°C, and then returned to anaerobiosis. Chloramphenicol (150 µg/ml) was added to block new protein synthesis during the periods of aeration and repair. At time zero, the cultures were restored to anaerobiosis, and the recovery of Edd activity was monitored. Edd activity before aeration was defined as 100%. GS09 (SOD$^-$/vector) (▲); GS10, (SOD$^-$/pyggX) (●).
Fig. 3.4. SoxRS is activated by redox-cycling drugs under anaerobic conditions if terminal oxidants are present. (A) Strain TN530 (soxS::lacZ) was grown aerobically in LB medium (◆); anaerobically in LB medium (■), in LB/nitrate medium (▲), or in LB/fumarate medium (●). Strain GS69 (narG soxS::lacZ) was grown anaerobically in LB/nitrate medium (△); strain GS70 (frd soxS::lacZ) was grown anaerobically in LB medium (□) or LB/fumarate medium (○). At an OD600 of 0.07, PQ was added, and the cultures were further incubated at 37 °C for one hour. (B) Strain TN530 (soxS::lacZ) was grown anaerobically in LB medium (■), or in LB/fumarate medium (●). Strain GS67 (nuo ndh soxS::lacZ) (◇) and GS68 (ubi men soxS::lacZ) (○) were grown anaerobically in LB/fumarate medium.
Fig. 3.5. The anaerobic electron-transport chain oxidizes the PQ radical. PQ (5 μM) reduced by dithionite (30 μM) under anaerobic conditions displayed an absorption maxima at 395 nm (solid line). When both inverted vesicles and fumarate (5 mM) were added to the above mixture, the absorption maxima at 395 nm disappeared (dashed line). The presence of either vesicles or fumarate alone did not cause any change (not shown, for clarity).
Fig. 3.6. By serving as an artificial electron sink, ferricyanide enables anaerobic PMS to activate SoxRS. Strain TN530 (soxS::lacZ) was grown anaerobically in LB medium to an OD$_{600}$ of 0.05. Where indicated, PMS was added to a final concentration of 25 µM, and potassium ferricyanide was added to final concentrations of 150 µM or 800 µM. All cultures were further incubated anaerobically at 37 °C for one hour.
Fig. 3.7. The [2Fe-2S] cluster of SoxR is directly oxidized by redox-cycling drugs in vivo and in vitro.  
(A) Strain XA90/pKOXR containing the soxR expression plasmid was grown in anaerobic LB medium to an OD$_{600}$ of 0.15. IPTG was added, and the cultures were incubated anaerobically at 37 °C for 1.5 hr. Where indicated, PQ was then added, and cultures were further incubated anaerobically at 37 °C for 30 min. EPR samples from these intact cells were prepared as described in Experimental procedures. The peaks indicated by arrows represent the EPR signal contributed by reduced SoxR; they are absent from vector control strains (not shown).  
(B) Purified SoxR protein (15 µM) was reduced with dithionite anaerobically. Excess dithionite was removed (see Materials and Methods). Reduced SoxR was then treated with 25 µM PMS in anaerobic buffer at 25 °C for 2 min. For EPR sample preparation, see Experimental procedures.
Fig. 3.8. The inducing effect of paraquat does not depend on its inhibition of the SoxR reduction systems. (A) Strains TN530 (wt soxS::lacZ) (●) and SP14 (zwf pnt soxS::lacZ) (■) were cultured in anaerobic LB medium to an OD$_{600}$ of 0.10. PQ was added, and the cultures were further incubated anaerobically at 37°C for 45 min. (B) Strains TN530 (wt soxS::lacZ) (●), GS63 (rseC soxS::lacZ) (■), GS64 (rsxC soxS::lacZ) (▲) and GS65 (rseC rsxC soxS::lacZ) (◆) were cultured in anaerobic LB/nitrate medium to an OD$_{600}$ of 0.10. PQ was added, and the cultures were further incubated at 37°C for 45 min.
Fig. 3.9. Absorbance spectra of purified SoxR proteins. SoxR (3 µM) was isolated in an oxidized form (solid line); it could be reduced under anaerobic conditions by dithionite (dashed line).
Fig. 3.10. SoxR does not inhibit cytochrome c reduction by $O_2^-$. Purified SoxR protein was reduced by dithionite anaerobically, and excess dithionite was removed (see Materials and Methods). 4 μM of cytochrome c, 5.4 mU/ml of xanthine oxidase and 8 or 24 μM of reduced SoxR were added to the assays before the addition of 20 μM xanthine. The reduction of cytochrome c was monitored by its absorbance at 550 nm.
Fig. 3.11. PMS is toxic to *E. coli* under anaerobic conditions. GC4468 (wild type) was cultured anaerobically to an early log phase in glucose/nitrate medium supplemented with aromatic, branched-chain, and sulfur-containing amino acids. PMS was added at time zero, and cell growth was monitored thereafter. ●, no drug treatment; ◆, 30 μM PMS; ▲, 120 μM PMS.
Fig. 3.12. PMS blocks the synthesis of branched-chain amino acids in anaerobic cells, and the SoxRS response is protective. Wild type strain (GC4468, panel A) and soxRS mutant (DJ901, panel B) were cultured to early log phase in anaerobic glucose/nitrate medium supplemented with aromatic and sulfur-containing amino acids. Where indicated, branched-chain amino acids (ILV) were included. At time zero, PMS was added and growth was monitored.
Fig. 3.13. Redox-cycling drugs directly inactivate [4Fe-4S] fumarases. (A) Resox drugs inactivate fumarase B anaerobically in vivo. Strain SJ33 (fumCA) was grown anaerobically in LB medium. When OD$_{600}$ reached 0.2, 150 µg/ml chloramphenicol was added to stop protein synthesis. Where indicated, 200 µM PQ, 300 µM MD or 30 µM PMS was added. All cultures were further incubated at 37 °C anaerobically for 45 min. (B) PMS directly inactivates purified fumarase A in vitro. Purified fumarase A was incubated anaerobically on ice by itself (●) or with 0.5 µM (▲), 1 µM (◆) or 5 µM (■) PMS. At time points, damage was terminated by the addition of 20 mM L-malate, and fumarase A activities were measured.
Fig. 3.14. (A) Malate protects fumarase A from PMS. Purified fumarase A was exposed to 10 μM PMS in the presence of malate in 0 °C anaerobic buffer. After 3 min of anaerobic incubation, fumarase activity was measured. (B) PQ-inactivated fumarase A can be reactivated by reconstruction of its iron-sulfur cluster. Purified fumarase A was incubated with 1 μM PQ in anaerobic buffer at room temperature for 3 min. L-malate (20 mM) was then added to stop damage. Enzyme activity was determined immediately or following cluster repair. Damaged [3Fe-4S] clusters were rebuilt by the addition of Fe(NH₄)₂(SO₄)₂ and DTT; clusters that had degraded beyond that state were rebuilt by the addition of Fe(NH₄)₂(SO₄)₂, DTT, cysteine, and purified IscS.
3.7 References


CHAPTER 4: CONCLUSIONS

4.1 Summary of current work

4.1.1 Superoxide inactivates mononuclear iron enzymes by enabling mismetallation \textit{in vivo}.

Superoxide (O$_2^-$) is a toxic byproduct of aerobic metabolism. In response, \textit{E. coli} has evolved to have two cytosolic O$_2^-$-scavenging enzymes called superoxide dismutases (SODs) (20, 33). \textit{E. coli} strains that lack both cytosolic SODs (SOD$^-$ mutants) fail to grow aerobically without supplements of either branched-chain amino acids or aromatic amino acids (7). The branched-chain amino acid auxotrophy has been shown to be due to inactivation of [4Fe-4S] cluster dehydratases (9-11).

However, there are no such dehydratases in the aromatic amino acid biosynthetic pathways, which implies the existence of other cellular targets for O$_2^-$ \textit{in vivo}. \textit{E. coli} strains unable to scavenge hydrogen peroxide exhibit aromatic amino acid auxotrophy that resembles what we have seen in the SOD$^-$ mutants. Therefore, we wondered if H$_2$O$_2$ and O$_2^-$ have the same targets in the aromatic biosynthetic pathways. Work in our lab has shown that the mononuclear iron enzymes are target(s) of H$_2$O$_2$ (1, 28). These enzymes require a divalent metal at the active site for activity. Since they use ferrous iron \textit{in vivo}, they are potential targets of oxidants, such as H$_2$O$_2$ and O$_2^-$. DAHP synthase, the first enzyme in the aromatic biosynthetic pathways, is a mononuclear iron enzyme.

We found that DAHP synthase is damaged in the SOD$^-$ mutants cultured aerobically, and we confirmed that this enzyme is directly inactivated by O$_2^-$ \textit{in vitro}. Our
observations opened up the possibility that \( \text{O}_2^- \) may inactivate this type of mononuclear iron enzyme in general. Therefore, we selected three enzymes from this family for inspection -- threonine dehydrogenase (Tdh), ribulose-5-phosphate 3-epimerase (Rpe) and peptide deformylase (PDF). These enzymes catalyze different categories of chemical reactions and are involved in diverse metabolic pathways. All three enzymes were shown to be inactivated by \( \text{O}_2^- \) both \textit{in vivo} and \textit{in vitro}. The inactivation rate constants were comparable to those with which \( \text{O}_2^- \) reacts with \([4\text{Fe}-4\text{S}] \) dehydratases. It was initially a puzzle how \( \text{O}_2^- \) could damage those iron enzymes \textit{in vivo}, since unlike \( \text{H}_2\text{O}_2 \) which can form a ferryl radical with \( \text{Fe(II)} \) and damage the polypeptide, \( \text{O}_2^- \) is not able to do so. In fact, we found that Tdh and Rpe isolated from the \( \text{O}_2^- \)-stressed stain were metallated with \( \text{Zn(II)} \) rather than with \( \text{Fe(II)} \). Therefore, we propose that \( \text{O}_2^- \) oxidizes \( \text{Fe(II)} \), the oxidized \( \text{Fe(III)} \) quickly dissociates from the enzyme, and \( \text{Zn(II)} \) gets the chance to bind. Since \( \text{Zn(II)} \) binds to these enzymes much more tightly than does iron, it traps the enzymes in the less efficient mismetallated forms.

4.1.2 The SoxRS response is directly activated by redox-cycling drugs rather than by superoxide.

The SoxRS regulon of \textit{E. coli} is induced by in the presence of redox-cycling drugs through a two-step cascade of gene activation (14, 30). SoxR is the redox sensor, which is activated when its 2Fe-2S cluster is oxidized (18). It is conserved in Proteo- and Actinobacteria. Redox-cycling drugs generate superoxide, besides Mn-superoxide dismutase was identified as a member of the SoxRS regulon, therefore superoxide was thought to be the inducer of SoxRS. However, we showed that toxic levels of superoxide accumulated in a superoxide-dismutase-deficient strain failed to effectively
activate SoxRS. Redox-cycling drugs activated SoxRS anaerobically, and respiratory substrates –fumarate, nitrate and oxygen- progressively stimulated SoxRS induction by these drugs. This suggests that superoxide is not the signal that SoxR senses, and that the key role of oxygen and other respiratory substrates is to regenerate the oxidized drugs, which trigger SoxR oxidation. Indeed, we showed that redox-cycling drugs oxidized the 2Fe-2S cluster in SoxR anaerobically both in vivo and in vitro, while superoxide could not effectively oxidize SoxR in vitro. SoxRS affects the expression of nearly 100 genes (6), most of which do little to ameliorate superoxide toxicity. Instead, they focus upon reducing the intracellular levels of redox-cycling drugs. Drugs are widely present in nature. Besides generating superoxide, they may directly oxidize 4Fe-4S dehydratases, deplete cellular NADPH pool, and form Michael adducts with cellular nucleophiles. We showed that SoxRS induction protected E. coli against drugs anaerobically, which supports the idea that it is useful to evolve an anti-drug defense which does not depend upon superoxide as a signal and functions in anaerobic environments.

4.2 Possible future work

4.2.1 Why are SOD⁻ mutants aromatic amino acid auxotrophs?

Cells stressed with O₂⁻ and H₂O₂ are auxotrophs of aromatic amino acids. O₂⁻ was thought to cause the aromatic auxotrophy by oxidizing the intermediate of the transketolase reaction (5, 29). We did observe that transketolase lost over half of its activity in O₂⁻–stressed cells. However, one might expect that a loss of 50% enzyme activity would not cause severe damage to the pathway it belongs to. Moreover,
manganese supplementation relieves the aromatic auxotrophy in both O$_2^-$ and H$_2$O$_2$–stressed cells, suggesting that O$_2^-$ and H$_2$O$_2$ are likely to cause the auxotrophy in a similar manner. We showed that DAHP synthase, the first enzyme in the aromatic biosynthetic pathway, is damaged by O$_2^-$. This enzyme has also been shown to have substantially low activity in H$_2$O$_2$–stressed cells (Jason Sobota, unpublished data). Therefore, DAHP synthase is the common target that O$_2^-$ and H$_2$O$_2$ share in the aromatic biosynthetic pathway.

Overexpression of DAHP synthase fails to fully restore growth of an SOD$^-$ mutant in the absence of aromatic amino acids, which implies the possible existence of other targets in that pathway. In fact, 3-dehydroquinat synthetase, which catalyzes the reaction following DAHP synthase, is annotated to be a zinc-dependent enzyme (22, 23, 31). However, a number of iron-dependent enzymes were purified with zinc bound, due to the fact that zinc is relatively abundant in cells and it binds to proteins tightly. Therefore, it would be worthwhile checking what metal 3-dehydroquinate synthetase actually uses in vivo, and if the enzyme loses activity in O$_2^-$–stressed cells.

4.2.2 Can other soft metals inactivate mononuclear iron enzymes?

Soft metals are functionally defined by their polarizability, which enables them to bind tightly to sulfhydryl groups. Mercury(II), silver(I), copper(I), lead(II), cadmium(II), nickel(II), zinc(II), and cobalt(II) are well studied soft metals, and their toxicities to bacteria are roughly in proportion to their affinities for sulfur (26). This suggests that enzymes containing key sulfhydryl moieties are likely to be the cellular targets of soft metals. Work done in our lab has shown that Cu(I), Ag(I), Hg(II), Cd(II), and Zn(II)
attack the exposed [4Fe-4S] clusters in dehydratases (24, 32). We found that overload of zinc causes mismetallation of mononuclear iron enzymes in the absence of oxidative stress. Then it will be interesting to test if other divalent soft metals, such as Hg(II) and Cd(II), can also poison these mononuclear iron enzymes in vivo and in vitro.

4.2.3 Are mononuclear iron enzymes targets of redox-cycling drugs?

We showed that the SoxRS response system is induced by redox-cycling drugs even under anaerobic conditions (15). Aside from making reactive oxygen species, we found that these drugs poison E. coli by directly oxidizing the [4Fe-4S] clusters in dehydratases (15). Both [4Fe-4S] cluster dehydratases and mononuclear iron enzymes employ a solvent-exposed iron atom to help with catalysis, which can be oxidized by univalent oxidants, such as O$_2^\cdot$. Therefore, it is possible that redox-cycling drugs may similarly oxidize the catalytic iron in mononuclear iron enzymes and inactivate them. To test this idea, one might check if mononuclear iron enzymes lose activities upon drug treatment under anaerobic conditions. If so, the impact of drugs on purified enzymes can be tested.

4.2.4 Does superoxide demetallate Fur?

The ferric uptake regulator (Fur) senses the intracellular free iron concentration and regulates gene expression accordingly. When E. coli is replete with iron, Fur binds Fe$^{2+}$ and acquires the activity as a repressor of multiple iron-import genes, whereas when E. coli starves for iron, Fur is demetallated and the iron import systems are activated to satisfy the organism’s demands for iron (16, 17). Therefore, the expression levels of
iron-import genes reflect the metallation status of Fur. Fur-regulated genes are repressed in the wild type strain, indicating that Fur is metallated with Fe\(^{2+}\). However, the same genes are fully expressed in the SOD\(^{-}\) strain, which suggests that O\(_2\)^\(-\) disrupts the metallation of Fur and thereby its ability to properly regulate genes.

To test this idea directly, one might purify the Fur protein, load it with ferrous iron, and then monitor its DNA-binding ability upon O\(_2\)^\(-\) challenge by fluorescence anisotropy DNA-binding assay.

### 4.2.5 How are O\(_2\)^\(-\)-sensitive enzymes repaired \textit{in vivo}?

O\(_2\)^\(-\) inactivates [4Fe-4S] cluster dehydratases and mononuclear iron enzymes so fast (\(\sim 10^6 – 10^7\) M\(^{-1}\) s\(^{-1}\)) that it is likely that even wild-type cells contain enough superoxide to continually damage these enzymes. In response, bacterial cells have acquired the ability to repair the damaged enzymes. In fact, when superoxide-stressed cells were returned to anaerobiosis, the damaged dehydratases and mononuclear iron enzymes quickly restored their activities in the absence of new protein synthesis. The half-time of their repair is on the minute scale (13, 21).

The mechanism of repair remains unknown. In the case of dehydratases, conversion of a [3Fe-4S]\(^{1+}\) cluster back to the active [4Fe-4S]\(^{2+}\) form requires both reduction and metallation. Reduction probably happens first so that the unstable [4Fe-4S]\(^{3+}\) intermediate may be avoided. The damaged clusters can disintegrate beyond the [3Fe-4S]\(^{1+}\) form \textit{in vivo} when stress is prolonged (8). In that case, cluster repair might utilize the same pathway that originally assembles the cluster in newly synthesized proteins. This process is accomplished in \textit{E. coli} by proteins in either the Isc system or
the Suf system (2, 12). However, it has been shown that: 1) only the iscS mutant exhibited a substantial defect in cluster repair, while all isc genes are important for \textit{de novo} cluster assembly; 2) \textit{E. coli} mutants lacking Suf proteins repaired clusters at normal rates. Therefore, the mechanism of cluster repair under mild oxidative stress condition is distinct from that of \textit{de novo} assembly.

Recent studies have suggested that YggX, a member of the SoxRS regulon, is involved in cluster repair (27). The \textit{yggX} null mutation slowed the recovery of dehydratase activities in SOD\textsuperscript{-} mutants upon return to anaerobiosis. And also, overproduction of \textit{yggX} enabled SOD\textsuperscript{-} mutants to fully restore dehydratase activities and at rates similar to the wild type strain. The repair mechanisms of YggX remain unclear. There are also other small proteins, such as YtfE (19), which have been proposed to be involved in cluster repair. However, direct evidence is absent.

In the case of mononuclear iron enzymes, cells need to remove the mischarged zinc ion and then recharge the enzyme with iron. The rate limiting step might be the removal of zinc, since zinc binds to these enzymes very tightly. For instance, the half time of zinc dissociation from Rpe is over 8 hours (28). However, Rpe successfully completes its shift from Zn\textsuperscript{2+} to Fe\textsuperscript{2+}-metallated form within only 60 min of return to anaerobiosis. This implied that there exist cellular components which assisted in pulling zinc out of the enzyme. We showed that a physiologically relevant concentration of cysteine (~300 µM) is able to chelate zinc out of Rpe within an hour \textit{in vitro}. This suggests that cysteine is a possible candidate which may help to pull zinc out of those mismetallated mononuclear enzymes and thereby facilitate their repair \textit{in vivo}. To test if cysteine is responsible for \textit{in vivo} repair, a \textit{ΔcysA} mutation that prevents assimilation of sulfate from medium can be introduced into the SOD\textsuperscript{-} strain. This new strain can be grown aerobically with the
supplementation of cysteine. Cells can then be centrifuged and resuspended in anaerobic medium without cysteine. The recovery of Rpe and Tdh activities can be monitored thereafter. Besides cysteine, redoxin-type proteins, including glutaredoxins and thioreductions, could also plausibly mediate zinc extraction from mismetallated mononuclear enzymes.

4.2.6 How does *E. coli* keep the cysteine residue in PDF reduced *in vivo*?

PDF employs a cysteine residue to coordinate the catalytic metal ion at the active site (3, 4). We found that molecular O$_2$ oxidized this cysteine residue in the Fe-charged PDF but not in the apo-form of PDF. Addition of TCEP, a thiol-containing reductant, was able to prevent Fe-PDF from oxidation. This suggests that molecular O$_2$ can oxidize the sulphydryl through the catalysis of iron.

However, in wild type cells cultured aerobically, PDF is metallated with iron but its cysteine residue is kept reduced. This indicates the existence of cellular reductants which help to keep the cysteine residue from oxidation. Possible candidates include glutaredoxins, thioredoxins and glutathione. To understand how PDF is kept reduced in vivo, each of these candidates might be knocked out, and the oxidation status of PDF cysteine residue may then be tested.

There are four glutaredoxins and three thioredoxins. Knocking out any of the glutaredoxins or thioredoxins seems to induce the expression of other glutaredoxins or thioredoxins (25). Besides, it does not seem feasible to knock all of them out at once. These make it hard to examine their roles in keeping PDF reduced *in vivo*. Aside from
these candidates, there may be other unknown cellular reductants which help to keep the cysteine residues reduced.
4.3 References


APPENDIX A: THE ROLE OF YGGX IN PROTECTION AGAINST SUPEROXIDE STRESS

The yggX gene encodes a small and abundant protein with a molecular weight of 11 kDa. Sequence comparisons revealed that yggX homologs are conserved throughout gram-negative bacteria and that the structural organization of its three neighboring genes (mutY – yggX – mltC – nupG) is also conserved (5). Notably, the mutY gene encodes a DNA glycosylase involved in repair of oxidative DNA damage (4).

Mutations in the yggX gene were originally isolated during a genetic screen for Thi+ revertants from a gshA− strain of Salmonella enterica (5). Mutants that overproduce the YggX protein showed the suppression of several phenotypes related to oxidative damage, including deficiency in aconitase activity, elevated spontaneous mutation rate, and hyper-sensitivity to paraquat (5). The same group has also reported that YggX decreased chelatable iron in solution and protected DNA from iron-mediated oxidative damage (6). These observations supported a model in which YggX protects labile [4Fe-4S] clusters and therefore reduces iron-mediated DNA damage.

In E. coli, yggX is under the control of SoxRS (15). The transcriptional regulation of yggX is consistent with its proposed role in the protection of oxidant-sensitive [4Fe-4S] clusters. The structure of YggX has been solved via nuclear magnetic resonance (NMR). However, Fe(II) binds only weakly to the protein, and not to a highly conserved region of the structure, which suggested that the role of YggX in iron trafficking might be more complex than previously thought (2).
In this study, we showed that artificial induction of *soxS* resulted in an increase in 6-phosphogluconate dehydratase (Edd) activity in O$_2$-stressed cells (Fig. A1). Edd contains a [4Fe-4S] cluster that is vulnerable to O$_2^-$. Induction of *yggX* was also able to increase Edd activity to a similar level (Fig. A1). When *yggX* was deleted, however, *soxS* induction no longer had any effect on Edd activity (Fig. A1). These data indicated that *yggX* is the only gene under SoxRS control that helps to keep labile [4Fe-4S] cluster dehydratases active under O$_2^-$ stress. Further, we found that YggX facilitated repair of these [4Fe-4S] cluster dehydratases in O$_2$-stressed cells (Fig. A2), while it did not help with *de novo* Fe-S cluster synthesis, since the activities of oxidant-resistant Fe-S cluster enzymes – NADH dehydrogenase I and succinate dehydrogenase – were not affected by *yggX* induction (Fig. A3).

How does YggX facilitate Fe-S cluster repair? One possibility could be that YggX sequesters Fe(II) and provides it to the cluster repair process. If this hypothesis were true, then one might expect that *yggX* overproduction would result in decrease in intracellular free iron level, which would cause a reduction of iron-mediated DNA damage. In fact, we found that *yggX* overproduction decreased mutagenesis of SOD$^-$ mutants by ~100-fold (Table A1). Furthermore, elevated level of YggX decreased H$_2$O$_2$-mediated death of SOD$^-$ mutants, which is also consistent with the idea that YggX limited the amount of free iron available for Fenton chemistry. We then measured intracellular unincorporated iron levels in SOD$^-$ mutants with or without *yggX* overproduction. However, we found that *yggX* induction only moderately reduced iron levels under O$_2^-$ stress (Fig. A5), which raised the puzzle whether indeed YggX can sequester Fe(II).

Overall, although there are several pieces of evidence which are consistent with the idea
that YggX is involved in iron trafficking, direct evidence is still absent and its role in iron binding might be more complicated than previously thought.

### A.1 Methods

**Construction of plasmids overproducing yggX and soxS.** To create plasmids overexpressing yggX, the yggX ORF was PCR-amplified from *E. coli* GC4468 by using the forward primer 5′- ACTGC\textbf{GAATTCTAGCGGCTCCCGTGAG} and the reverse primer 5′- TCAC\textbf{CTCTAGAGGAGATGAGCAACGCG}. The EcoRI and XbaI sites are underlined. PCR products were digested and cloned into pCKR101 vector behind a tac promoter to generate the pyggX plasmid. The insert was verified by sequencing. The psoxS plasmid was made using the same method. The forward primer was 5′- GTGAC\textbf{GAATTCCGCGAATACCGAC} and the reverse primer was 5′- TCAC\textbf{CTCTAGAGCGAAGGCGATGCCGC}.

**Enzyme assays.** Cell extracts were prepared by either sonication or passage through a French press. Total protein content was determined using the Coomassie Blue dye-binding assay (Pierce). The activity of 6-phosphogluconate dehydratase (Edd) was assayed by the two-step procedure for determination of pyruvate (3). NADH dehydrogenase I (Ndh I) activity was measured by the oxidation of deaminoNADH (13), which was oxidized by Ndh I but not Ndh II. Succinate dehydrogenase activity was determined by plumbagin-dependent cytochrome c reduction (8).
**H₂O₂-mediated killing.** Cells were cultured anaerobically in LB medium to an OD₆₀₀ of 0.1 with or without *yggX* induction. To induce *yggX* expression, 100 µM IPTG was added. Cultures were then shifted to aerobic environments and aerated at 37°C for 30 min. Cells were adjusted to 0.025 OD₆₀₀, and 2.5 mM H₂O₂ was added. At time points aliquots were removed and diluted into LB plus catalase. Further dilutions of the samples were added to LB top agar and were plated on LB. Colonies were enumerated after overnight incubation at 37°C. Survival is normalized to the number of viable cells prior to the addition of H₂O₂.

**thyA forward mutagenesis assay.** To determine the rate of mutagenesis, Thy⁻ mutations were selected using trimethoprim (14). Trimethoprim inhibits the growth of Thy⁺ cells but not that of *thyA* mutants if thymine is supplied in the growth medium. Cells were precultured to an early log phase anaerobically in LB media, which were then diluted in the same media and aerated at 37 °C. To maintain the cells in exponential phase, they were subcultured as necessary during aeration. To induce *yggX* expression, 100 µM IPTG was added. After 4 hr aeration, cells were diluted to an OD₆₀₀ of ~0.01 and plated anaerobically onto LB plates containing 1 mg/ml thymine and 0.1mg/ml trimethoprim to allow outgrowth of *thyA* mutants at 37 °C.

**EPR measurement of unincorporated intracellular iron.** Cells were grown in glucose/amino acids minimal A medium with or without *yggX* induction. At ~0.2 OD₆₀₀, cells were centrifuged and resuspended in 8 ml of medium prewarmed to 37 °C. 1 ml of 100 mM DTPA was added to block further iron import, followed by 1 ml of 200 mM
deferoxamine mesylate. The latter compound penetrate cells, binds unincorporated ferrous iron, and in the presence of oxygen triggers its oxidation to EPR-detectable ferric iron. Cells were aerated 37 °C for 15 min and then spun down. The cell pellet was washed twice with 5 ml of cold 20 mM Tris-HCl (pH 7.4), and then resuspended in ~200 µl of cold 20 mM Tris-HCl plus 15% glycerol, pH 7.4. An aliquot of the cell suspension was loaded into a quartz EPR tube and frozen on dry ice.

EPR spectra were obtained under the following conditions: microwave power, 10 mW; microwave frequency, 9.05 GHz; modulation amplitude, 12.5 Gauss at 100 KHz; time constant, 0.032; and sample temperature, 15 K. Iron concentrations were determined based on iron standards. Iron standards were prepared by making serial dilutions of FeCl₃ in 20 mM Tris-HCl containing 10% glycerol and 1 mM desferrioxamine. The iron concentrations of these standard solutions were determined using ε₉₃₃ at 420 nm of 2.865 cm⁻¹. Intracellular iron concentrations were calculated by normalizing the iron measurements to intracellular volume, using the conversion that 1 ml of 1 OD bacteria collectively contains 0.52 µl of cytosol (9).
Table A.1 *yggX* induction decreased mutagenesis of SOD\(^{-}\) mutants.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>wt</th>
<th>SOD(^{-})</th>
<th>SOD(^{-}) <em>yggX</em></th>
<th>SOD(^{-}) <em>yggX/yggX</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutants per 10(^{7}) cells</td>
<td>16</td>
<td>1011</td>
<td>1034</td>
<td>9</td>
</tr>
</tbody>
</table>

*E. coli* strains were precultured to an early log phase anaerobically in LB media, which were then diluted in the same media and aerated at 37 °C. To maintain the cells in exponential phase, they were subcultured as necessary during aeration. After 4 hour aeration, the cultures were diluted to an OD\(_{600}\) of ~0.01 and plated anaerobically onto LB plates containing 1 mg/ml thymine and 0.1 mg/ml trimethoprim to allow outgrowth of thyA mutants.
Fig. A.1. yggX induction increased 6-phosphogluconate dehydratase activity in O$_2^-$-stressed cells. Anaerobic cultures of SOD-deficient strains in LB plus 0.2% gluconate were diluted into the aerobic medium to an OD$_{600}$ of 0.05. 100 µg/ml ampicillin and 100 µM IPTG were included. The cultures were grown at 37°C with vigorous aeration. At ~0.4 OD$_{600}$, cells were harvested for the measurement of 6-phosphogluconate dehydratase (Edd) activity. Edd activity in a wild type strain was defined as 100%.
Fig. A.2. YggX accelerated the repair of [4Fe-4S] cluster dehydratases damaged by superoxide.
SOD-deficient strains were grown anaerobically to an OD_{600} of 0.20 in LB medium supplemented with 0.2% gluconate, 100 µg/ml ampicillin and 100 µM IPTG. Cells were shifted to aerobic environments, aerated for 30 min at 37 °C, and then returned to anaerobiosis. Chloramphenicol (160 µg/ml) was added to block new protein synthesis during the periods of aeration and repair. At time zero, the cultures were restored to anaerobiosis, and the recovery of Edd activity was monitored. Edd activity before aeration was defined as 100%.
Fig. A.3. YggX did not have an impact on de novo synthesis of Fe-S clusters. Anaerobic cultures of SOD-deficient strains were diluted into aerobic LB medium to an OD$_{600}$ of 0.05. 100 µM IPTG was added to induce expression of yggX. The cultures were grown at 37°C with vigorous aeration. At ~0.4 OD$_{600}$, cells were harvested for the measurement of NADH dehydrogenase I (Ndh I) activity (A) and succinate dehydrogenase (Sdh) activity (B).
Fig. A.4. *yggX* induction decreased H$_2$O$_2$-mediated death in SOD$^-$ mutants. Cells were cultured anaerobically in LB medium to an OD$_{600}$ of 0.1. 100 µM IPTG was added to induce expression of *yggX*. The cultures were then shifted to aerobic environments and aerated at 37°C for 30 min. The cultures were adjusted to ~0.025 OD$_{600}$ and were challenged with 2.5 mM H$_2$O$_2$. Challenges were terminated by dilution into LB plus catalase. Cells were plated onto LB in 3 ml of top agar and incubated anaerobically for overnight growth.
Fig. A.5. \textit{yggX} induction moderately decreased intracellular free iron levels in $\text{O}_2^-$-stressed cells. Cells were pre-cultured in minimal A medium containing 0.2% glucose and 0.5 mM L-amino acids. Then the cultures were diluted into 1 L of the same medium aerobically. 100 µM IPTG was added to induce expression of \textit{yggX}. The cultures were grown at 37°C with vigorous aeration. At ~0.2 OD\textsubscript{600}, cells were harvested for EPR measurements.
APPENDIX B: HOW DOES SUPEROXIDE DISRUPT FUR METALLATION?

*E. coli* has two cytosolic superoxide dismutases (SODs), MnSOD (encoded by *sodA*) (10) and FeSOD (encoded by *sodB*) (17). We found that *E. coli* lost over 80% of FeSOD activity when treated with paraquat (PQ) (Fig. B1), which generates $O_2^-$ as well as other oxidants inside cells. To deduce which product made by PQ diminishes FeSOD activity, we grew wild type and *sodA* mutant in the presence and absence of PQ, and then we ran their crude extracts on the SOD activity gel. In the *sodA* mutant, PQ killed almost all of the FeSOD activity, while in the wild type strain, FeSOD activity seemed unaffected (Fig. B2). In the presence of PQ, the SoxRS response is activated, which turns on the expression of *sodA*. Therefore, FeSOD activity was saved when MnSOD was induced to scavenge $O_2^-$. We thus concluded that $O_2^-$ diminished FeSOD activity.

Then how does $O_2^-$ block FeSOD activity? There are two possibilities: 1) the FeSOD protein is not correctly metallated, since iron is strictly required by this enzyme for catalysis; 2) $O_2^-$ might decrease FeSOD expression level. To test the possibility of mismetallation, we partially denatured FeSOD prepared from cell extracts, removed whatever metals from the active sites, and then put back extra amount of iron. In the untreated cells, all of the FeSOD proteins were correctly metallated with iron (Fig. B3). However, in PQ-treated cells, a small fraction of the enzyme was not correctly metallated (Fig. B3). Therefore, the decrease in FeSOD activity is the result of both mismetallation and down regulation.

RyhB is the only known regulator of *sodB*. It is a small anti-sense RNA which triggers degradation of *sodB* mRNA (11). A *ryhB* null mutation plus reconstitution fully
restored FeSOD activity to a PQ-treated sodA strain (Fig. B4 A). Therefore, O$_2^-$ decreased sodB expression level via RyhB. Our RT-PCR data further confirmed that O$_2^-$ led to decrease in sodB RNA level and increase in RyhB RNA level (Fig. B4 B). The response E. coli to PQ / O$_2^-$ is largely controlled by the SoxRS system (7, 16). We showed that deletion of soxS plus reconstitution fully restored FeSOD activity to a PQ-treated sodA strain (Fig. B5), which suggested that SoxRS induction is necessary to block FeSOD activity. However, artificial expression of soxS from a plasmid in the absence of PQ did not affect FeSOD activity, indicating that SoxRS induction is not sufficient to block FeSOD activity.

Then what else besides including SoxRS can PQ / O$_2^-$ do to raise RyhB RNA level? Fur is the possible connection between SoxRS and RyhB (11). Since O$_2^-$ causes FeSOD mismetallation, then is it possible that O$_2^-$ also causes Fur de-metallation, which allows RyhB to be accumulated? We first showed that repression of sodB by O$_2^-$ requires the function of Fur. If we knocked out the SoxS binding site of fur, we no longer saw the accumulation of RyhB RNA or the repression of sodB RNA upon PQ treatment (Fig. B6 A). We also constructed a plasmid expressing a mutated version of Fur, which has Leu in place of the His residue in the Fe-binding site of Fur and therefore cannot bind Fe. We cultured the sodA’ fur’ in LB medium, where Fur is bound by iron and is active in repressing ryhB. When we turned on the expression of the mutated Fur on the plasmid, the Fur regulon was de-repressed (Fig. B6 B), and FeSOD activity was diminished (Fig. B6 C). This implied that apo-Fur diluted out the normal Fe-bound Fur, and the mix-dimer was not active in repressing ryhB.

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To summarize, our model is shown as below: PQ turns on the SoxRS response, which makes a number of Fur proteins. Meanwhile, $O_2^-$ demetallates Fur, and apo-Fur allows RyhB RNA to be accumulated, which then degrades $sodB$ mRNA.

**B.1 Methods**

**Enzyme assays.** Cell extracts were prepared by either sonication or passage through a French press. Total protein content was determined using the Coomassie Blue dye-binding assay (Pierce). β–galactosidase activity was assayed as described (14). SOD activity was assayed by the xanthine oxidase-cytochrome c method (12).

**SOD activity gel.** SOD activity gel assays were performed following the method developed by Beauchamp and Fridovich (1).

**Reversible constitution of FeSOD.** To remove metals from FeSOD, the crude extracts were first incubated with 0.2 M sodium carbonate buffer at pH 11 containing 2 mM EDTA and 5 mM DTT for 25 min at 30 °C, followed by dialyzing against 0.2 M sodium carbonate buffer at pH 9.2 containing 1 mM EDTA and 1 mM DTT for 7 hr at 4 °C. Then the extracts were dialyzed against 50 mM KPi (pH 7.8) containing 0.5mM DTT for 18hr at 4 °C. To put iron back to the enzyme, it was incubated with 50 mM KPi (pH 7.8) containing ferrous ammonium sulfate and DTT for 12 hr at 25 °C; and then dialyzed against several changes of KPi (pH 7.8). Purified FeSOD was used as a control – all activity was lost upon demetallation and was fully restored after reconstitution.
Paraquat (PQ) diminished FeSOD activity in a *sodA* mutant. The *sodA* mutant was cultured in LB medium. Where indicated, 200 µM of PQ was added to the growth medium. The cultures were grown at 37°C with vigorous aeration. At ~0.3 OD$_{600}$, cells were harvested for the measurement of FeSOD activity. FeSOD activity with no PQ treatment was defined as 100%.
Fig. B.2. O$_2^-$ caused decrease of FeSOD activity. Wild type strain (WT) and sodA$^-$ mutant were cultured in LB medium. Where indicated, 200 µM of PQ was added to the growth medium. The cultures were grown at 37°C with vigorous aeration. At ~0.3 OD$_{600}$, cells were harvested for SOD activity gel assays.
Fig. B.3. $\text{O}_2^-$ resulted in mismetallation of FeSOD. The sodA mutant was cultured in LB medium. Where indicated, 200 µM of PQ was added to the growth medium. The cultures were grown at 37°C with vigorous aeration. At ~0.3 OD$_{600}$, cells were harvested for FeSOD activity measurement and metallation status determination. “recons.” represents FeSOD activity after dimetallation and recharge with Fe.
Fig. B.4. O$_2^-$ decreased $sodB$ expression level via RyhB. (A) FeSOD activity; (B) RyhB RNA level. Cells were cultured in LB medium. Where indicated, 200 µM of PQ was added to the growth medium. The cultures were grown at 37°C with vigorous aeration. At ~0.3 OD$_{600}$, cells were harvested for FeSOD activity measurement and metallation status determination. "recons." represents FeSOD activity after dimetallation and recharge with Fe.
**Fig. B.5.** SoxRS induction was necessary but not sufficient to block FeSOD activity. Cells were cultured in LB medium. Where indicated, 200 µM of PQ was added to the growth medium. To induce soxS expression, 100 µM IPTG was added. The cultures were grown at 37°C with vigorous aeration. At ~0.3 OD<sub>600</sub>, cells were harvested for FeSOD activity measurement and metallation status determination. “recons.” represents FeSOD activity after dimetallation and recharge with Fe(II).
Fig. B.6. (A) Repression of sodB by PQ required the function of Fur. Cells were cultured in LB medium. Where indicated, 200 µM of PQ was added to the growth medium. fur(NI) is the fur non-inducible strain which lacks the SoxS-binding site in the fur promoter region. At ~0.3 OD₆₀₀, cells were harvested for FeSOD activity measurement. (B) Expression of apo-Fur resulted in Fur regulon de-repression. The sodA mutant was transformed with either an empty vector or the vector cloned a mutated version of Fur, which has a Leu in place of the His in the Fe-binding site. To induce the expression of the mutated Fur, 500 µM IPTG was added. The levels of iucC::lacZ expression were measured to reflect the metallation status of Fur. 1 mM dipyridyl was added as a control to show the fully expressed level of iucC::lacZ. (C) Expression of apo-Fur blocked FeSOD activity. Cells were also harvested for FeSOD activity measurement.
REFERENCES OF APPENDICES A & B


