IDENTIFICATION AND CHARACTERIZATION OF A NOVEL CLASS OF HYDROGEN PEROXIDE TARGETS IN *ESCHERICHIA COLI*

BY

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DISSERTATION

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ABSTRACT

In nature, microorganisms are constantly exposed to micromolar levels of hydrogen peroxide (H_2O_2) from sources such as host defenses or byproducts of chemical processes. This constant threat raises two major questions: what biomolecules are damaged by H_2O_2, and how do cells defend themselves against it? Using *Escherichia coli* strains that are devoid of peroxide scavengers (Hpx^-), I was able to study the effects of protracted, low-grade H_2O_2 stress.

In this study, a novel class of H_2O_2-sensitive enzymes is described. I discovered that Hpx^- cells were unable to metabolize gluconate via the pentose phosphate pathway. By assaying activity of various enzymes in the pathway, one protein, ribulose 5-phosphate 3-epimerase (Rpe), which requires a solvent-exposed divalent metal for activity, was found to be sensitive to H_2O_2 both in vivo and in vitro. Although Zn, Mn, Fe, and Co were found to activate Rpe to varying degrees, only Rpe loaded with Fe was sensitive to H_2O_2, which suggests that Fe is the metal that activates Rpe under normal conditions. In addition, Mn supplements were found to protect Rpe from damage by H_2O_2 in vivo, probably by replacing Fe in the active site.

Growth studies also revealed that Hpx^- cells are auxotrophic for aromatic amino acids. Less than 0.3 μM H_2O_2 is necessary to cause this phenotype, making it the most sensitive pathway known. This auxotrophy can be suppressed by adding shikimic acid, an intermediate in the common pathway for aromatic amino acid synthesis (the shikimic acid pathway), as well as Mn^{2+}. I have found that the first enzyme in the shikimic acid pathway (DAHP synthase), which has been shown to have the ability to use Fe^{2+} as a cofactor, loses activity in Hpx^- cells grown in the presence of oxygen. This failure is due to accumulation of damage to DAHP synthase by a Fenton reaction between H_2O_2 and the active-site Fe atom. This damage results in pathway failure and an inability to synthesize aromatic compounds. The cell attempts to compensate by inducing DAHP synthase transcription, but this is not enough. Mn^{2+} can replace the Fe in the protein, allowing growth, but still cannot fully activate DAHP synthase to wild-type levels.

Thus, Fenton chemistry remains the sole mode of H_2O_2 toxicity currently known, but the scope of targets of the Fenton reaction is increasing, as many proteins, such as Rpe and DAHP synthase, may use Fe as a mononuclear metal cofactor. Certain aspects of protection against damage by H_2O_2 are becoming clearer. While the OxyR response increases production of H_2O_2 scavenging enzymes, it also plays a role in metal homeostasis through regulation of MntH and Dps. These proteins work to increase Mn concentrations, while sequestering Fe in the stressed cell, respectively. This process allows Mn to metallate and thereby protect enzymes which would otherwise be vulnerable to damage by H_2O_2. Therefore, adjusting metal availability is an important defense mechanism against H_2O_2, given that enzymes that use divalent metals are significant targets. Each mononuclear Fe-containing enzyme studied thus far could be protected from oxidation by addition of Mn^{2+}, which may help to explain why cells induce Mn import during H_2O_2 stress.

To my family

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CHAPTER 1: INTRODUCTION

1.1 A BRIEF EVOLUTIONARY HISTORY OF LIFE

1.1.1 In the beginning

When life first appeared on Earth some 3.8 billion years ago, the environment was very different than exists today. With an atmosphere devoid of molecular oxygen, early organisms would have encountered copious amounts of ferrous iron and plenty of sulfur [3, 5, 83]. Other bio-relevant metals such as cobalt, manganese, nickel, and molybdenum were most likely available, though at several orders of magnitude lower in concentration than iron. Two other biologically relevant metals, copper and zinc, were extremely scarce during the early evolution of life, as they probably existed as precipitates with organic and inorganic sulfides [3, 196]. These differences in relative availability of metals and profusion of sulfur led early metabolic pathways to be built using these elements, both due to both their abundance and the wide array of chemical reactions that iron and sulfur are able to facilitate [186, 187]. In fact, organisms today show evidence of these early preferences, such that prokaryotes require more iron, manganese, and cobalt and much less zinc relative to eukaryotes, which evolved much later and under very different conditions [49].

1.1.2 The rise of oxygen

Life would continue to evolve under these anoxic, iron-rich conditions for the next billion years. Over this time period basic metabolic processes were fine-tuned using iron as an important cofactor, both as a stand-alone facilitator of difficult reactions, as well as a partner of sulfur in iron-sulfur cluster proteins [83]. Thus, iron became deeply entrenched in metabolism from the very beginning [164]. Should the earth have remained anoxic, these metabolic processes would have continued to be adequate to

sustain life. However, the evolution of photosystem II would prove to challenge this lifestyle.

The first oxygen-producing photosystems appeared between 2.4 to 2.7 billion years ago [102, 110]. While it would take almost a billion years for oxygen to begin to accumulate in the atmosphere, it most likely began oxidizing ferrous iron as it was produced. This titration limited atmospheric oxygen level for some time, but made reduced iron species harder for organisms to acquire from the environment. Because iron had already established a firm foothold on various metabolic processes [164], it was much easier for early life to evolve new ways to obtain iron, such as siderophores and other iron import systems, rather than to rebuild metabolism from the bottom up. This did not mean, however, that early organisms could not use oxygen as a tool in their metabolic repertoire.

In fact, there is some evidence that early life was able to begin taking advantage of oxygen before the appearance of photosystem II, over 2.9 billion years ago. LUCA, or the Last Universal Common Ancestor of Eukaryotes, Archaea, and Bacteria, is thought to have already evolved a cytochrome oxidase [28]. This early cytochrome oxidase likely evolved from NO reductase, which was part of the denitrification pathway. Other evidence of early aerobic metabolic processes includes the predicted presence of oxygen-dependent pyridoxal synthetic enzymes predating the appearance of photosystem II [99].

These processes required molecular oxygen in order to function, so why would they have appeared before photosystem II? Oxygen could have been generated by the organisms themselves by enzymes such as methane monooxygenase found in the strict anaerobe *Methylomirabilis oxyfera* [51]. It is also possible that some oxygen was generated by the UV irradiation of glacial ice, causing local accumulation of hydrogen peroxide (H_2O_2) in meltwaters, which was then converted into molecular oxygen and water [100]. The presence of a mangano-catalase roughly 3 billion years ago both suggests the presence of H_2O_2 and provides a mechanism for production of molecular oxygen without photosystem II [99]. Some even suggest that catalase enzyme was the precursor to manganese center of the photosynthetic oxygen-evolving complex [18].

However, there is still debate as to whether these processes really occurred before photosystem II, as increasing evidence suggests replacement of alternative anaerobic reactions with more kinetically favorable aerobic enzymes over evolutionary history [148].

1.1.3 Problems associated with oxygen

The Great Oxygenation Event yielded a boon in aerobic metabolic processes. It has been suggested that the presence of molecular oxygen in the atmosphere enabled more than 1000 new and unique metabolic reactions [149]. These oxygen-dependent enzymes were preferred over their anaerobic counterparts due to their thermodynamic efficiency and irreversibility. New metabolites, including steroids, were also made possible by the accumulation of oxygen [93]. These aerobic metabolites tend to be less polar, making it easier for them to cross membranes. However, most good things come with a price. While oxygen yielded a plethora of new routes to create an array of metabolites, it brought with it new challenges that life would have to overcome in order to benefit from this new tool.

1.1.3.1 Changes in metal availability

One major challenge to early life upon accumulation of molecular oxygen was a change in the availability of biologically relevant metals. The geologic record suggests a two-step change in metal availability [152]. The first major change, which occurred between 2.4 and 1.8 billion years ago, resulted in significant drops in soluble levels of iron and cobalt, with a slight drop in soluble manganese levels [196]. These drops were accompanied by a significant increase in the availability of molybdenum [157]. These were only the first set of changes that life would face with respect to metal availability.

The second major change in soluble metal concentrations occurred about 800 million years ago. This change saw levels of iron, manganese, and cobalt fall further, while levels of molybdenum and nickel rose significantly [157, 196]. While there was no appreciable change in levels of copper or zinc during the first major event, this round was very different. The organic sulfides that had kept these metals as precipitates became

oxidized by the rise in molecular oxygen levels. This released huge amounts of copper and zinc causing the soluble levels of these two metals to skyrocket.

Despite this sudden availability of copper and zinc, life had already established its metabolic processes with metals such as iron, manganese, and cobalt. Early organisms had a choice to make: completely overhaul metabolism, or develop new ways to acquire the metals they had long been accustomed to. Because changing the entire suite of metabolic processes would entail tweaking many different enzymes which carried out a number of different types of reaction, it was much easier to solve the problem by evolving ways of obtaining metals that had become scarce. These acquisition systems, if effective, would allow all metabolic processes requiring a particular metal to function without the need to redesign each enzyme individually.

1.1.3.2 Generation of reactive oxygen species

Coping with a decrease in the availability of necessary metals was not the only challenge brought on by the accumulation of molecular oxygen. While molecular oxygen itself is a rather weak oxidant, partially reduced oxygen species are much better electron acceptors (Reaction 1.1). These reactive oxygen species (ROS), which include hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot -}$), and the hydroxyl radical ($HO\cdot$), have reduction potentials well suited to oxidize various biomolecules, thereby damaging an array of cellular targets [135].

$$O_2 \xrightarrow[-0.16]{e^-} O_2^{\cdot -} \xrightarrow[+0.94]{e^-,\ 2H^+} H_2O_2 \xrightarrow[+0.38]{e^-,\ H^+} HO\cdot \xrightarrow[+2.33]{e^-,\ H^+} H_2O \quad \text{(Reaction 1.1)}$$

It was not immediately obvious, however, that these reactive oxygen species were toxic. In fact, it was not until the discovery of dedicated scavenging systems that the effects of these oxidants were appreciated. The first of these enzymes to be discovered was by Oscar Loew in 1900. Loew observed a protein in a range of higher organisms that catalyzed the conversion of two molecules of H_2O_2 to molecular oxygen and water,

which he named “catalase” [118]. While Loew recognized that metabolic processes must generate H_2O_2 and that this might toxify the cell, he failed to develop any molecular mechanisms for these processes.

It was then nearly 70 years before superoxide dismutases (SODs) were discovered in the lab of Irwin Fridovich in 1969 [122]. As their name implies, SODs dismute two $O_2\cdot^-$ molecules into molecular oxygen and H_2O_2. Others took steps to explore the effects of $O_2\cdot^-$ on cells which lacked these scavengers, finding a number of processes that were injured by this reactive oxygen species [26, 31, 181].

Since then, a wide range of catalases, SODs, superoxide reductases, as well as peroxidases have been described in all domains of life, even obligate anaerobes [91, 118, 119, 129, 145]. Without these defenses, organisms would be unable to cope with the resulting onslaught of oxidation they would otherwise experience. The requirement for these scavenging enzymes during growth in oxygen has led to an entire field devoted to the elucidation of the effects of their respective substrates. The field of oxidative stress encompasses all aspects of reactive oxygen species, from how they are made, to what they damage, and how cells cope with their effects. Each of these areas will be discussed in turn as the major questions of the field of oxidative stress are explored.

1.2 MAJOR QUESTIONS IN THE FIELD OF OXIDATIVE STRESS

Much work has been done in the field of oxidative stress to understand the various reactive oxygen species and how they affect cells. There are four major questions that embody past, present, and future directions in oxidative stress research. While each of these areas will be described in greater detail later in this chapter, an overview of each will be given here as an introduction.

The first major question in oxidative stress is where do reactive oxygen species come from? This question can be explored from two distinct angles – sources external to the cell, and sources within the cell. For $O_2\cdot^-$, the source is important, as its charged

nature makes it difficult for this oxidant to cross membranes [193]. Therefore, the location of its generation will likely be the location of any targets. However, H_2O_2 can easily cross biological membranes, so the targets of H_2O_2 will be less dependent on the source of this oxidant [159].

This leads to the second major question in oxidative stress – what are the targets of reactive oxygen species? In the case of superoxide, solvent-exposed Fe-S clusters are major targets in a number of organisms [17, 65, 111, 116, 183, 188]. While other phenotypes, such as auxotrophies for sulfur-containing, and aromatic amino acids have been described [26], there is a lack of strong evidence in the literature with regard to the cause of either. Suppression of these auxotrophies by mutations in *dapD* or *fur* imply a role for iron, but the specific targets are yet to be identified [120]. In the case of H_2O_2, the Fenton reaction, which will be discussed later, is the common theme. Oxidation of sulfhydryls and lipid peroxidation have also been suggested as mechanisms of H_2O_2 damage, but these avenues are not likely factors in *Escherichia coli* [194]. Details of specific phenotypes will be described later.

The third major question in the field of oxidative stress is how do organisms cope with reactive oxygen species? This question follows directly from the first two, as an understanding the sources and targets of reactive oxygen species is integral to comprehending the strategies employed to overcome them. These strategies include responding to the reactive oxygen species themselves, reacting to and repairing damage, and changing metabolism to circumvent the damage altogether.

Finally, another point of consideration in oxidative stress, but by no means the least important, is what are the evolutionary and ecological impacts of reactive oxygen species? From ongoing evolutionary battles, to occupation of particular ecological niches, to the very existence of obligate anaerobes, the roles of reactive oxygen species remain important factors.

1.3 WHERE DO REACTIVE OXYGEN SPECIES COME FROM?

1.3.1 External sources of ROS

As was discussed in the previous section, there are two categories of major sources of reactive oxygen species. The first category to be discussed includes those sources external to the cell. In each of these cases, the reactive oxygen species is acting on internal targets, but the primary source of the oxidant is extracellular.

One such source of reactive oxygen species is the immune response. In plants, NADPH oxidases at wound sites produce vast amounts of $O_2^{\cdot -}$ and H_2O_2 in order to defend them against invading microorganisms [11, 124]. NADPH oxidases are also present in mammalian macrophages and neutrophils, where these systems operate in concert with generation of reactive nitrogen species, release of antimicrobial peptides, and sequestration of metals to kill invading microbes [63, 147]. It has been established that organisms unable to defend themselves against $O_2^{\cdot -}$ generated by the immune response are attenuated [108, 109], although the role of H_2O_2 in the oxidative burst remains unsolved [10, 173].

Some organisms, either because they are unable to mount an immune response, or as a secondary means of protection, employ redox-cycling warfare as a strategy to compete with and defend against microorganisms in their vicinity. Plumbago, a flowering shrub, generates the redox-cycling drug plumbagin, which is an organic compound that generates reactive oxygen species by stealing electrons from metal centers and flavins once the drug has been taken up by other organisms [73, 180]. Juglone, a compound very similar in structure and function to plumbagin, is used by black walnut trees to defend against microorganisms, as well as insects and other plants [88]. Bacteria such as *Streptomyces* and *Pseudomonas* species, as well as *Pantoea agglomerans*, use phenazine compounds such as pyocyanin to defend against microbial competitors [179]. All of these drugs work because they easily enter the cytoplasm and readily accept electrons from various sources as promiscuity makes defending against them more difficult.

Some organisms resort to producing H_2O_2 directly. One such organism, the bombadier beetle, has a specialized gland which produces H_2O_2 [40]. In this chamber, H_2O_2 is quickly combined with catalases, peroxidases, and hydroquinones to produce a hot spray as a defense mechanism against larger predators. Other organisms, such as lactic acid bacteria, are more subdued in their approach. These bacteria use pyruvate-, lactate-, and NADH oxidases that produce as much as millimolar amounts of H_2O_2, which is released into their surroundings as a means to compete with other organisms in their environment [66, 162, 170].

Not all sources of reactive oxygen species are biological. Indeed, chemically-generated species can be formed by a variety of reactions. For example, illumination of riboflavin or other chromophores is a common source of reactive oxygen species in lab medium [24]. Ascorbate is also commonly added to medium to generate reactive oxygen species. In the environment, catechols from rotting plants can generate reactive oxygen species, as can reduced sulfur species released from anaerobic sediments which can, in a metal-dependent manner, produce reactive oxygen species when they rise to the oxygenated layer.

1.3.2 Internal sources of ROS

While there are a number of external sources of reactive oxygen species that organisms must contend with, it turns out that, in the absence of defense systems, cells produce enough oxidants to damage themselves without help from their environment. Indeed, when scavenging systems are removed from cells, a number of growth phenotypes have been observed. These will be discussed later [26, 89, 140, 184]. However, these reactive oxygen species are accidental byproducts of aerobic metabolism, not desired end products or useful intermediates.

It was initially suspected that H_2O_2 and $O_2^{\cdot -}$ were merely inadvertent products stemming from partial reduction of oxygen by cytochrome oxidase in the respiratory chain. However, this idea did not hold up to experimental scrutiny [127]. Another idea was that flavoproteins or quinones in the respiratory chain were the primary contributors

to intracellular generation of reactive oxygen species. This theory was plausible, as in vitro experiments had demonstrated the ability inverted membrane vesicles from bacteria and mitochondria generated H_2O_2 and $O_2^{\cdot -}$ during in vitro respiration [30, 85, 125]. Indeed, other experiments showed reactive oxygen species generation in vitro by two respiratory chain enzymes, NADH dehydrogenase II and succinate dehydrogenase [126]. However, these ideas were also shown to be incorrect, as *E. coli* NADH dehydrogenase mutants did not decrease the rate of production of H_2O_2 or $O_2^{\cdot -}$, and cells overproducing this protein had only a slight increase in production of reactive oxygen species [160].

While the thought that the respiratory chain was the primary source of H_2O_2 proved to be false, the idea that flavoproteins were responsible was not. While the few flavoproteins found in processes outside the respiratory chain and most flavoenzymes do not contribute to H_2O_2 formation in a significant way, one such protein proved to be a major player under certain growth conditions. NadB, which is a flavin-dependent dehydrogenase, was found to be responsible for nearly 30% of the total H_2O_2 produced endogenously during aerobic growth [104]. NadB typically uses fumarate as an anaerobic electron acceptor during biosynthesis of nicotinamide. However, when cells are growing aerobically, oxygen can serve as an alternative electron acceptor, resulting in H_2O_2 production. This process seems to be tolerated by cells because the flux through this particular pathway is usually very low. Additionally, NadB is rather unique, as most other flavoproteins with significant flux, such as sulfite reductase, glutamate dehydrogenase, and alkylhydroperoxide reductase, do not employ flavins as the terminal redox moiety. This arrangement allows them to pass electrons to other carriers, such as disulfides or heme, rather than to oxygen. This setup drastically decreases the lifetime of the electrons on flavins, thereby preventing the adventitious reaction with oxygen that would otherwise lead to H_2O_2 production. Fumarate reductase, another flavoprotein similar to NadB, can also generate fair amounts of H_2O_2 [104]. This is another unique case, however, as fumarate reductase is only expressed under anaerobic conditions, so generation of H_2O_2 by this enzyme would be limited to a window of time immediately following a shift from anaerobic to aerobic growth conditions.

Another source of H_2O_2 that has been discovered is autoxidation of menaquinone [103]. This process can account for up to an additional 10% of the total intracellular production of H_2O_2. However, the sources of more than half of the total H_2O_2 generated during aerobic growth are yet to be determined. While it is possible that a single predominant source will someday be found, it is far more likely that many processes contribute small amounts of H_2O_2 toward the total, making those individual processes harder to locate.

1.4 WHAT ARE THE TARGETS OF ROS?

1.4.1 Targets of $O_2\cdot^-$

As a radical species, it might seem that $O_2\cdot^-$ might readily oxidize a host of biological targets. However, due to electrostatic repulsion, electron-rich targets inhibit abstraction of electrons by the anionic $O_2\cdot^-$ [16, 52, 82, 155]. It was reported in 1976 that *E. coli* experienced a number of growth defects when exposed to hyperbaric oxygen [20]. These phenotypes, specifically deficiencies for aromatic, branched-chain, and sulfur-containing amino acids, were explored further and found to be the result of $O_2\cdot^-$ stress by using superoxide dismutase (SOD) mutants [26]. This finding shows that despite the limits on its interactions with certain biomolecules, $O_2\cdot^-$ is still able to cause damage to cells. Each of the three auxotrophies will be discussed in turn.

First, the branched-chain amino acid auxotrophy was found to result from damage to a particular class of iron-sulfur (Fe-S) cluster enzymes, known as dehydratases [54, 111]. These dehydratases are characterized by a solvent-exposed [4Fe-FS] cluster in which one iron atom is not fully coordinated but is left with one coordination site available to bind to and activate substrate. While the dehydratase responsible for the branched-chain amino acid auxotrophy was found to be dihydroxyacid dehydratase (DHAD), another enzyme in the same pathway, isopropylmalate isomerase (IPMI) is also a dehydratase sensitive to $O_2\cdot^-$ oxidation. In fact, there are at least six additional proteins

fitting this description, including aconitase A and B, fumarase A and B, 6-phosphogluconate dehydratase (Edd), and serine dehydratase [55, 59, 60, 78, 116]. In each of these cases, the exposed cluster reacts with $O_2^{\cdot -}$, which univalently oxidizes the cluster, causing the iron atom to fall out and the cluster to be degraded (Reactions 1.2 and 1.3). This occurs with second-order rate constants of between 10^6 and 10^7 M^{-1} s^{-1} [54].

$$[4Fe\text{-}4S]^{2+} + O_2^{\cdot -} + 2H^+ \longrightarrow [4Fe\text{-}4S]^{3+} + H_2O_2 \qquad \text{(Reaction 1.2)}$$

$$[4Fe\text{-}4S]^{3+} \longrightarrow [3Fe\text{-}4S]^{1+} + Fe^{2+} \qquad \text{(Reaction 1.3)}$$

The second phenotype, an auxotrophy for cysteine and methionine, is yet to be explained. It has been observed that unidentified sulfur compounds are exported from the cell, but it is unclear how this explains the deficiency [13]. Due to damage to Fe-S clusters, the demand for cysteine, which is used to build the clusters, may be higher, but this hypothesis has not been tested directly. The ability of a *dapD* mutations (which causes cells to accumulate dipicolinic acid, a metal chelator), as well as deletion of *fur* (ferric uptake repressor), to relieve this phenotype does propose a role for iron, but no concrete answer has been determined [120].

The third phenotype, which can also be relieved by mutations in *dapD* and *fur*, is the aromatic amino acid auxotrophy [120]. This phenotype has been suggested to result from damage to the glycolate intermediate formed during the transketolase reaction [12]. The resulting oxidation product would cause the enzyme to become stalled in an inactive state until the glycolate adduct was lost. It is unclear if, or how quickly, this resolving reaction would occur. One important note is that this theory was tested using hyperbaric oxygen in wild-type strains rather than by using SOD mutants. It is not clear that this is the actual cause for the aromatic amino acid auxotrophy caused by $O_2^{\cdot -}$ in vivo. Further work is necessary to fully explain this phenotype as well.

1.4.2 Targets of H_2O_2

1.4.2.1 The Fenton reaction

Unlike the charged $O_2^{\cdot -}$ radical, H_2O_2 can easily penetrate cellular membranes [159]. In principle, H_2O_2 could act as both a univalent and divalent oxidant; however, the high energy of activation associated with breaking the dioxygen bond makes H_2O_2 unable to react with most biomolecules. Despite this, a bolus of H_2O_2 added to a wild-type *E. coli* culture will immediately stop growth, and protracted, low-level exposure leads to a loss in viability. This damage is not due to direct interaction of H_2O_2 with biomolecules but is mediated by the Fenton reaction (Reaction 1.4).

$$Fe^{2+} + H_2O_2 \longrightarrow [FeO^{2+}] + OH^- + H^+ \longrightarrow Fe^{3+} + OH^- + HO\cdot \quad \text{(Reaction 1.4)}$$

The Fenton reaction is the basis for all known modes of H_2O_2 toxicity in *E. coli*. As shown in reaction 1.4, this reaction is dependent on the presence of reduced iron. The initial reaction between the H_2O_2 and reduced iron produces a ferryl radical intermediate, $[FeO^{2+}]$. The lifetime of this intermediate is unknown, but it is possible that this species may dissociate away from the site of its formation decomposing to produce the highly-reactive hydroxyl radical ($HO\cdot$). A powerful oxidant, $HO\cdot$ can react at diffusion-limited rates with virtually any biomolecule.

1.4.2.2 DNA damage

One of the most consequential phenotypes observed as a result of H_2O_2 stress is DNA damage. H_2O_2 cannot itself react with DNA; however the negative charge of the sugar-phosphate backbone attracts cations, such as iron, to its surface. Iron may also interact with bases via π orbitals. Here, H_2O_2 participates in the Fenton reaction on the face of DNA, producing $HO\cdot$ [75]. The $HO\cdot$ then immediately oxidizes the DNA, resulting in formation of DNA radicals which are subsequently resolved into a variety of different types of lesions [47, 81]. These lesions accumulate and eventually leave the cell

unable to replicate, resulting in a filamentation phenotype [140]. If the cells are unable to carry out recombinational repair, these lesions ultimately lead to cell death [27, 41, 86].

One question regarding the extremity of this phenotype was the reaction rate associated with the Fenton reaction. The published rate constant for ferrous iron with H_2O_2 was 76 M^{-1} s^{-1} [189]. This rate is much too slow to explain the damage incurred by cells, as the half-time of the reaction using the physiologically relevant steady-state concentration value for H_2O_2, around 20 nM, is about five days. However, this calculation was made at pH 3 using hexaqueous iron. When the rate was measured at a physiological pH with iron liganded to DNA or ATP, the rate constant increased to 2000 – 6000 M^{-1} s^{-1} [140]. This rate is sufficient to explain the high levels of DNA damage that result from H_2O_2 stress.

Evidence that DNA damage by H_2O_2 results from the Fenton reaction in vivo is provided by the finding that cell-permeable chelators, such as desferrioxamine, are able to rescue cells. This phenotype can also be rescued by overexpression of Dps, which is a ferritin-like iron sequestration protein induced during H_2O_2 stress. Alternatively, when either *fur* or *dps* is deleted, resulting higher intracellular levels of iron causes an increase in DNA damage [140]. In addition, cells lacking the YaaA protein, which is involved in controlling iron levels, also show DNA-damage phenotypes; although the actual mechanism by which this protein acts is unknown [117]. Together, these findings make a strong case that iron is integral to the toxicity of H_2O_2 with respect to DNA.

1.4.2.3 Protein and membrane damage

Iron serves an integral role in DNA damage by H_2O_2, but is the same true for proteins and membranes? With this in mind, one obvious target of H_2O_2 to consider is Fe-S cluster proteins, specifically those dehydratases with solvent-exposed clusters. Here, the Fenton reaction would occur between H_2O_2 and the tri-coordinate iron atom of the exposed cluster. When this class of enzymes was examined, they were indeed found to be sensitive to H_2O_2, with inactivation rate constants in the range of $10^3 – 10^4$ M^{-1} s^{-1} [89]. Some phenotypes related to damage to these dehydratases include an inability to grow on TCA cycle intermediates, likely due to damage to clusters in aconitase and

fumarase, and if additional H_2O_2 is added, an auxotrophy for leucine, caused by damage to two proteins in the biosynthetic pathway, dihydroxyacid dehydratase (DHAD) and isopropylmalate isomerase (IPMI) [89].

The need for higher H_2O_2 concentrations to obtain a visible phenotype likely derives from the ability of substrate to protect the enzyme. As damage occurs and activity of the enzyme decreases, substrate accumulates. Eventually, substrate will be able to compete with H_2O_2 for the active site, allowing the protein to turn over [89]. This cannot be achieved without the additional ability of the cell to repair damaged clusters. If cluster damage were irreversible, new protein would have to be synthesized de novo. This task would become difficult in the case of the leucine auxotrophy, as there would be no leucine available to synthesize new protein. In addition, de novo cluster assembly comes under fire, as the Isc (Iron sulfur cluster) assembly system is also sensitive to H_2O_2 [90].

Aside from Fe-S proteins, another iron protein examined was Fur, the ferric uptake repressor. The Fur regulon was found to be derepressed during H_2O_2 stress, implying that the reduced iron needed to activate Fur was not available [184]. It is unclear whether iron bound to Fur is oxidized, or if the phenotype is due to simply to lower concentrations of available reduced iron. No matter what the actual mechanism might be, derepression of the Fur regulon has dire implications for the cell. Some proteins under the control of Fur include iron transporters, which become induced during H_2O_2 stress. This leads to an increase in intracellular levels of iron, which as was mentioned before, contribute to higher levels of DNA damage. Thus, it is important for the cell to try to limit this response, which is why Dps in induced to sequester iron, and Fur itself is induced in order to shut down the regulon.

Another proposed protein target is oxidation of sulfhydryls. While it is possible for sulfhydryls to be oxidized by H_2O_2, the plausibility of this actually occurring in vivo is very low, as there are only a few examples of proteins with highly reactive thiols. The rate constant for H_2O_2 oxidation of a free thiolate anion does not exceed 20 M^{-1} s^{-1} [194] and similar values for the oxidation of active-site cysteine residues have also been

reported [36, 42, 69, 165]. Thus even at 1 μM intracellular H_2O_2, the half-time of the reaction would be nearly 10 hours – far too long to cause problems for the cell [82]. It is possible that oxidation of sulfhydryls might become important if the sulfur atom is involved in coordination of an iron atom. In this case, formation of the ferryl radical intermediate of the Fenton reaction might be able to oxidize the sulfhydryl, thereby quenching the radical, preventing $HO\cdot$ formation, and producing a sulfenic acid. Sulfenic acid species are easily reduced by the cell, so these lesions would not cause irreversible damage to the protein.

The final consideration for protein damage with respect to H_2O_2 stress is protein carbonylation. It is known that hydroxyl radicals can lead to carbonylation, which is elevated during exposure to redox-cycling compounds and H_2O_2 [48]. In addition, a much higher incidence of protein carbonylation is observed in *E. coli* mutants that cannot scavenge H_2O_2 [4]. In fact, when this is coupled with a mutation in Dps, resulting in higher levels of iron along with elevated levels of H_2O_2, carbonylation levels are extremely high. These facts imply that the Fenton reaction is also involved in generation of protein carbonyls, but the mechanisms remain unknown.

Another injury commonly proposed to result from H_2O_2 stress is lipid peroxidation [9, 64, 163]. While this mechanism of damage does seem to happen in higher organisms and some bacteria, the process appears to require polyunsaturated fatty acids. Since *E. coli* and most other bacterial lipids contain only saturated and monounsaturated fatty acids in their membranes [136], this form of damage does not seem to be relevant [15]. One notable exception is *Borrelia burgdorferi*, which is an intracellular pathogen that acquires fatty acids from its host, including polyunsaturated fatty acids. Indeed, *B. burgdorferi* did exhibit vulnerability to lipid peroxidation while *E. coli* did not [21]. Thus, while an important consideration in other systems, lipid peroxidation will not be addressed further with respect to *E. coli*.

1.4.2.4 The aromatic amino acid auxotrophy

The phenotypes discussed thus far occur in *E. coli* mutants in *ahpCF*, *katE*, and *katG*, which are devoid of H_2O_2-scavenging activity, and given the name Hpx^-, or

hydroperoxidase-deficient [158]. However, each of these phenotypes requires either rich medium, additional mutations, or exogenous H_2O_2, in order to be clearly visible [89, 90, 117, 140, 184]. However, there is one phenotype that is present in minimal glucose medium without addition of H_2O_2 or the need for additional mutations. This phenotype, an auxotrophy for the three aromatic amino acids (phenylalanine, tryptophan, and tyrosine), also occurs during $O_2\cdot^-$ stress [26, 89]. In the case of $O_2\cdot^-$ stress, damage to transketolase, as was described earlier, is prescribed to be the cause [12]. While the chemistry required for $O_2\cdot^-$ to oxidize the transketolase reaction intermediate is plausible, there is no involvement of iron in this mechanism, which is implied by the ability of a Δ*dapD* mutation to suppress the auxotrophy. All known mechanisms of H_2O_2 toxicity are mediated by the Fenton reaction and implicitly require iron, so it is unclear how transketolase would also be damaged by H_2O_2 and responsible for the phenotype observed. While it is possible to contrive a series of reactions for oxidation of the transketolase intermediate by H_2O_2, the likelihood that both oxidants are acting on the same protein by the same mechanism seems low because of difference in the mechanisms of oxidation. The mechanism of damage by $O_2\cdot^-$ would require univalent oxidation of the intermediate, while the mechanism of H_2O_2 damage would involve a divalent oxidation. Elucidation of the cause of this auxotrophy is one of the goals of this work and will be discussed later.

1.5 IRON IN PROTEINS

While numerous potential problems associated with the use of iron in aerobic environments have been discussed in previous sections, iron remains an important and integral part of aerobic life. In order to understand why life has continued to use this potentially vulnerable metal, it is also important to appreciate its uses in biological systems and prevalence in various processes. Exploration of these and other considerations regarding the maintenance of iron in aerobic metabolism will help to achieve this understanding.

1.5.1 Roles of iron

Iron is integrated into various aspects of metabolism, both aerobic and anaerobic. It exists in various forms and carries out a variety of functions. The role of iron with respect to metabolism, including structural elements it may be part of, fall into two major classes: redox reactions and non-redox applications. The specific role iron plays may differ within each category, but the type of reaction carried out is the distinguishing feature of each of these classes.

1.5.1.1 Redox reactions

One major role for iron in biological systems is to serve as a redox moiety. Many Fe-S cluster proteins serve this role. As the name implies, Fe-S cluster proteins have an absolute requirement for iron. No other metal is known to be able to substitute in a cluster. Typically, these clusters are buried within the polypeptide. The reduction potential of Fe-S clusters can range from -0.6 V to +0.45 V [25], making them suitable for a range of electron-transfer reactions, from ferredoxins to nitrogenases and hydrogenases [112].

Not all redox enzymes employ Fe-S clusters, but some use iron itself to carry out an array of functions. These proteins include heme-containing proteins like cytochrome oxidases or catalases, mononuclear enzymes like iron-dependent superoxide dismutases, binuclear ribonucleotide reductases, and 2-oxoglutarate-dependent oxidases. The ability of iron to convert between oxidation states allows these enzymes to carry out their respective redox reactions [35, 172, 182]. While other metals can also switch redox states, the redox potential is different for different metals. In fact the redox potential of a particular metal can be influenced by its ligands. While the particular function of each of these enzymes is different, they all have one thing in common – each has been tuned to a precise redox potential that requires a particular metal. Therefore, any changes in metal composition in these proteins could potentially disrupt their redox state and prevent them from carrying out the functions for which they were built [19, 121, 176].

1.5.1.2 Non-redox uses

The second role of iron in biological systems is in non-redox applications. These proteins themselves fall into two main categories. The first category includes proteins that require a metal for structure or proper positioning of cofactors. These proteins may exercise some degree of selectivity, but in most cases, these proteins can employ various metals with little to no change in activity. For example, methionine aminopeptidase [29], transketolase [171], and isocitrate dehydrogenase [132] have been shown to be active with a range of metals, such as cobalt, iron, manganese, nickel, and zinc.

Alternatively, many proteins require a metal for binding and activation of a substrate. This is analogous to the role played by the Fe-S cluster in dehydratases but can also be achieved using mononuclear metal centers. In this case, size, coordination state, and geometry play a crucial role in the ability of the metal to carry out the function. While some proteins may be able to use a number of metals, the activity of the protein is often changed as a result of metal substitution, as was the case with ribulose 5-phosphate epimerase (Rpe) [1]. Therefore, if the protein has evolved to use iron, substitution with other metals may lead to decreased activity, if not inactivation of the protein.

1.5.2 Prevalence of iron in metabolism

While the importance of iron as a cofactor for various biological processes has been illustrated, the number of known Fe-S cluster, heme-containing, and redox enzymes is limited. Therefore, at face value, it may not seem as though iron is very prevalent in metabolism. However, mononuclear metallo-proteins may represent a grossly overlooked category of iron proteins, as was discussed with respect to non-redox uses of iron. While searching enzyme databases may only reveal a handful of proteins in *E. coli* that have been annotated as using iron as a cofactor, many studies may have missed the pervasiveness of this metal simply due to experimental design.

There are many proteins in *E. coli* annotated as using a number of metals for various applications, but one common theme is that when the proteins were studied, the experiments were carried out under aerobic conditions. While researchers may have tried

to add iron in its reduced form to activate their protein of choice, it is extremely difficult to ensure the iron remains reduced. Therefore, while studies attempted to metallate divalent cation-requiring proteins with reduced iron, they were likely using iron in the oxidized form, leading researcher to conclude that iron could not be used as a cofactor by these enzymes [1]. If these experiments had been done under anaerobic conditions, where iron is easily maintained in the reduced form, it is unclear how many of these proteins may have been able to use iron. Thus, iron may actually be able to serve as a cofactor to a much wider range of proteins than has been realized.

1.5.3 Susceptibility of iron proteins to ROS

While iron carries with it a number of benefits, including its ability to provide cofactors with a wide range of reduction potentials and its ability to change between multiple coordination geometries, it also poses potential problems for aerobic life. While Fe-S proteins are extremely useful metabolic tools, they make cells vulnerable to oxidative stress – an unavoidable occurrence in aerobic habitats.

Aside from Fe-S cluster proteins, any protein that uses iron as a cofactor or substrate, such as Fur, is also susceptible to interference from reactive oxygen species. It has been hypothesized that Fur might not only sense iron levels, but also serve as a sensor for manganese as well [143]. This idea suggests that Fur might serve as a divalent metal sensor, rather than one specific to iron. This also presents an interesting quandary: if manganese will substitute for iron here, why has life not evolved an iron-independent metabolism?

1.5.4 Why is iron maintained in metabolic processes?

The question of why life has not evolved iron-dependent metabolism is not a simple one to answer. First, as described above, there are a number of reactions that require Fe-S clusters, which have an absolute requirement for iron. Thus, if Fe-S clusters are required for some enzymes, iron cannot be completely replaced without evolving enzymes that can carry out the same reaction without the need for the cluster.

Interestingly, there is an example of this in *E. coli*. Fumarase, an enzyme in the TCA cycle, has three separate isozymes. The two main isoforms use Fe-S clusters, one under aerobic conditions and the other when cells are growing anaerobically. However, the third fumarase isoform does not require a cluster and is induced during oxidative stress to compensate for inactivation of the sensitive protein [144]. Even if this is not possible for all processes that require Fe-S clusters, the number of these proteins is limited and would only account for a small group of proteins that would be susceptible to damage. Why, then, does life not evolve iron-independence in other processes?

The answer to this question is multi-faceted. First, reactions catalyzed by iron are not so easily achieved by other metals. Despite some proteins having the ability to be promiscuous in their metal preferences [29, 132, 171], other proteins are fine-tuned to the redox potential of a particular metal [19, 121, 176]. This is also true for enzymes that require their metal cofactor to switch between different coordination geometries. While this is possible for metals other than iron, (for example, iron and manganese are both especially facile at ligand exchange) one must remember the conditions under which life evolved. When life first evolved, the environment was rich in reduced iron [3], and metabolism was built around this metal [164]. Processes and pathways were constructed relying on these rather unique properties of iron that could not be achieved, at least not with the same kinetics, by using another metal. Over time, iron became so deeply entrenched in metabolism that was much easier for life to evolve defense mechanisms to address issues arising as a result of the use of iron in aerobic environments rather than completely redesign metabolic processes that had existed for over a billion years. Despite this fact, some organisms have developed iron-independent metabolism. *Borellia burgdorferi* seems be free of any iron-containing enzymes [146] and *Lactobacillus acidophilus*, a lactic acid bacterium, completely lacks Fe-S dehydratases [34]. Meanwhile, *Streptococcus pneumoniae*, another lactic acid bacterium, has evolved not to use iron under conditions during which it is making H_2O_2 [170]. It is important to note that this iron independence comes with a cost, as these organisms do not achieve the same metabolism in an iron-free way; lacking the TCA cycle, the ability to respire, and the ability to synthesize branched-chain amino acids.

1.6 HOW DO ORGANISMS COPE WITH ROS?

In order for organisms to survive the constant threat posed by reactive oxygen species in aerobic habitats, life had to evolve defenses against the agents and the damage they cause. If this did not occur, cells were either forced to develop new enzymes that were resistant to oxidants but could achieve the same end as their sensitive counterparts, or they lost the pathways altogether. The following section relates some of the strategies *E. coli* and other organisms employ to survive in aerobic environments.

1.6.1 Responses to ROS

One way cells can tolerate the generation of reactive oxygen species is to induce a suite of enzymes to cope with the related stress. There are three major responses employed by model bacterial systems: the SoxRS response, the OxyR regulon, and the PerR system.

1.6.1.1 The SoxRS response

The SoxRS system was discovered concurrently by the Demple and Weiss labs to be responsible for the induction of manganese-superoxide dismutase (Mn-SOD) in response to redox-cycling compounds [68, 144, 177]. Induction of Mn-SOD had been observed by Hassan and Fridovich over a decade earlier [72]. The two-component system uses the sensor (SoxR), which has an oxidizable [2Fe-2S] cluster, to induce transcription of *soxS* [46, 61, 76]. The SoxS protein, in turn, activates transcription of a number of genes encoding proteins involved in detoxification (Mn-SOD), Fe-S cluster repair (YggX), flavodoxins, drug efflux and modification (AcrAB, MarAB, YgfZ, NfsA, and NfnB), iron homeostasis (Fur), DNA repair (endonuclease IV), some oxidant-resistant isozymes of fumarase (FumC) and aconitase (AcnA) [144]. This response regulon was thought to be specific to $O_2{\cdot}^-$, given the fact that Mn-SOD is a member of the regulon. However, inclusion of Mn-SOD in the regulon is true in enterics, but not in many other bacteria [43, 101, 138]. Therefore, some doubt was cast on the specificity of the SoxRS regulon for $O_2{\cdot}^-$.

Indeed, it was shown that the response to $O_2\cdot^-$ in particular by the SoxRS system was not nearly as great as its response to redox-cycling compounds themselves [65, 115]. Nevertheless, these compounds do cause the production of intracellular reactive oxygen species, including $O_2\cdot^-$, making the SoxRS response important under these stress conditions; however it is becoming increasingly apparent that this system is not specific to $O_2\cdot^-$, but to the redox-cycling compounds themselves [43, 70, 106, 166].

1.6.1.2 The OxyR regulon

During H_2O_2 stress, *E. coli* and many other bacterial species employ the OxyR response to cope the problems that arise. OxyR itself is a transcriptional regulator that has two sulfhydryls in its active site, one of which is tuned to be easily oxidized by H_2O_2. In fact, the OxyR response can be activated by as little as 100 nM H_2O_2 and at a rate of approximately 10^7 M^{-1} s^{-1}. Upon oxidation and formation of a stabilizing disulfide, OxyR activates transcription of genes involved in various processes [33, 113, 198]. H_2O_2-scavenging enzymes, such as catalase and peroxidase, are induced to remove the stress and keep H_2O_2 concentrations below damaging levels. Iron acquisition and metabolism proteins, such as YaaA, Dps, and Fur, are induced as well [199]. YaaA is involved in iron metabolism via a yet-to-be determined mechanism [117]; Dps, an iron storage protein, is induced to sequester iron [197]; while Fur is likely induced in order to increase repression of iron import [140]. Together, these scavenging and iron regulatory systems work in concert to limit the damaging effects of the Fenton reaction.

Iron is not the only metal with regulatory ties to H_2O_2 stress. Manganese uptake (MntH) proteins are also induced in an effort to protect the cell, the hypotheses and models of which will be discussed later in detail [98]. Other proteins induced include HemH (ferrochelatase), which is presumed to be necessary to help the cell meet the increased demand for heme resulting from induction of catalase. Thiol homeostasis proteins (glutaredoxins and thioredoxins) and Fe-S cluster synthesis and repair systems (Suf) are also induced to help the cell cope with H_2O_2 stress [199].

1.6.1.3 The PerR system

The PerR system, found in *Bacillus* and other bacteria, is analogous to the OxyR response in *E. coli*, but only with respect to its function as a response regulator during H_2O_2 stress [23]. PerR itself is a homolog not of OxyR but of the Fur protein. Like Fur, PerR acts as a repressor under normal conditions and is capable of binding either iron or manganese. During H_2O_2 stress, PerR is only responsive when in the iron-bound form, which is able to facilitate oxidation of a histidine residue in the active site. This histidine then incorporates an oxygen atom, thereby displacing the iron, which leads to dissociation of the PerR-DNA complex [114]. Members of the regulon are then able to be transcribed, including *perR* itself as a feedback mechanism.

Members of the *Bacillus* PerR regulon resemble those under OxyR control in *E. coli* [74]. These include peroxidase and catalase to scavenge H_2O_2, as well as a number of genes necessary for heme synthesis. Fur and a Dps homolog, MrgA, are also expressed, which work together with the scavenging enzymes to limit Fenton chemistry. Another regulatory protein, Spx, is under PerR control [74]. Spx itself regulates genes that appear to be important during thiol-specific oxidative stress, such as thioredoxins [50, 134, 150, 200]. One unique aspect of the PerR regulon is that it also induces zinc uptake, which does not occur in the *E. coli* response to H_2O_2 [58]. It has been suggested that *Bacillus* might use high concentrations of zinc to protect sulfhydryls when high concentrations of H_2O_2 are present.

1.6.2 DNA repair systems

Separate from the oxidative stress regulons, organisms also have inducible DNA repair systems. As mentioned previously, DNA damage can have more impact than some phenotypes associated with reactive oxygen species. While stress responses that eliminate oxidants and limit iron will prevent further rounds of Fenton damage from occurring, DNA lesions occurring before these responses can be implemented will persist if not specifically addressed.

To counteract the damage to DNA, two different repair strategies are employed. The first, base excision repair, uses formamidopyrimidine DNA glycosylase (MutM and Fpg) in concert with endonucleases IV and VIII to initiate the removal of oxidized DNA bases [92, 153, 174]. This system recognizes not specific lesions but general distortions to the DNA helix. Thus, some lesions may go unrecognized and persist through DNA replication, resulting in strand breaks. When this happens, the cell employs the second repair system as a back-up – recombinational repair. The importance of these systems is made obvious when mutations in both systems are constructed, as these strains are only fully viable under anaerobic conditions [86, 131].

1.6.3 Alternative metabolism

While induction of defense systems to protect against and address damage that results from oxidative stress is a useful and successful strategy, cells sometimes express resistant isozymes to carry out processes that are otherwise sensitive to reactive oxygen species. Eamples of these proteins include aconitase and fumarase in the TCA cycle and the Suf pathway for iron sulfur cluster biosynthesis. In the case of fumarase and aconitase, the normal aerobic isozymes of these proteins use Fe-S clusters, which are sensitive to reactive oxygen species. However, under stress conditions, cells employ non-sensitive counterparts [37, 116, 183]. It is unclear how the Suf systems protects the clusters it builds from reactive oxygen species, but it is induced during oxidative stress due to failure of the Isc iron sulfur cluster assembly system, which is sensitive to oxidation [62, 90, 133, 137, 175, 195]. Some organisms take this one step further. Rather than make proteins that are sensitive to oxidation or replace these with resistant isozymes, some bacteria cope with the prevalence of oxidative stress with the complete absence of sensitive processes [56, 146]. For some organisms, such as the lactic acid bacteria mentioned previously, absence of these processes means they are auxotrophic for metabolites like branched-chain amino acids and TCA cycle intermediates whose biosynthetic pathways employ Fe-S cluster proteins.

A third strategy that is employed to counteract the deleterious effects of reactive oxygen species is to shift from an iron-mediated metabolism to one that relies more

heavily on manganese [6, 7]. There are several strategies that the cell enlists to limit the amount of intracellular free iron during oxidative stress, but the cell cannot survive without an alternative. As a result, *E. coli* and other bacteria pair iron limitation with manganese import [96, 141]. While this strategy does not protect cells from DNA damage by reactive oxygen species, it is effective in protecting protein targets [4]. The end result is a shift in the relative concentrations of manganese and iron, which may influence the metallation state, and by extension, the vulnerability of metallo-proteins to oxidation. This can be observed in the extreme, as some organisms have evolved manganese dependent metabolic processes and achieved iron independence [34, 56, 146, 170].

Aside from direct replacement of iron in proteins, there have been several other hypotheses proposed with respect to how manganese might protect the cell from reactive oxygen species [2, 8, 32, 39, 79, 80, 87, 97, 151, 154, 161, 178]. Some believed that manganese import protected the cell by activating the manganese-containing superoxide dismutase, but this was proven to be incorrect [4]. Another popular idea was that manganese was being imported to scavenge reactive oxygen species directly, as was observed in vitro [14, 67, 80]. While this may be possible with $O_2{\cdot}^-$, the relative amount of $O_2{\cdot}^-$-scavenging activity is more than an order of magnitude lower than the SOD activity in unstressed cells, making this scheme implausible as a means of protection. With respect to H_2O_2, it was shown in *E. coli* that manganese does not influence scavenging of H_2O_2 whatsoever [4]. Therefore, there must be a different mechanism by which manganese is acting. The Daly lab has proposed a model for H_2O_2 protection that uses small-molecule chelates of manganese to scavenge H_2O_2 in *D. radiodurans* [38]. While it is possible that this organism employs a strategy that is not present in *E. coli*, more evidence is needed to substantiate this model.

Despite the finding that these particular models are incorrect or do not occur in *E. coli*, it remains true that manganese does protect cells from oxidative stress. Therefore, it is important to determine the possible mechanisms by which this occurs. One focus of this study is to determine the avenue by which manganese protects cells from H_2O_2.

1.7 WHAT ARE THE ECOLOGICAL IMPACTS OF ROS?

Ultimately, the study of oxidative stress, from how oxidants are made, to what biomolecules they damage and how cells defend against them leads to an investigation into how these factors come together in an ecological sense. The ability to use or to survive reactive oxygen species is an important aspect of the field of oxidative stress and offers a number of interesting topics to consider, including evolutionary battles between damage and defense, habitation of niche environments, and the existence of obligate anaerobes. These wider views of the impact of oxidative stress will help to provide a deeper understanding of the fundamental principles involved in the shaping of defenses and metabolic pathways to withstand the ever-present threat posed by reactive oxygen species.

1.7.1 Ongoing evolutionary battles

Ever since the world became aerobic, there has been an evolutionary battle to survive reactive oxygen species. As cells began to cope with the existence of these oxidants, other organisms developed ways to harness their power. While higher organisms developed immune responses with an oxidative burst, pathogenic organisms evolved ways to suppress them or to survive in spite of them. For example, *Salmonella* is able to inhibit NADPH oxidase in macrophages to minimize the oxidative burst [185]. *Borrellia burgdorferi*, the causative agent of Lyme disease, has taken a different strategy to evade the damage caused by reactive oxygen species. This bacterium has evolved a completely iron-independent, manganese-dependent metabolism which allows it to survive the immune response in its hosts [56, 146].

A close study of concurrent evolution of pathogens and hosts provides a number of examples of one organism gaining the upper hand while the other evolves a way to counteract it, be it by evolving new offensive weapons, such as release of oxidants or limitation of metal in the host, to new defense mechanisms and metal acquisition systems in the pathogen [95, 156, 168, 191]. An understanding of the responses of both organisms with respect to components of oxidative stress may provide new insights into

particular targets of oxidation, as well as the importance of particular pieces of both host and pathogen defenses.

Survival in the host is not the only evolutionary battle between organisms with reactive oxygen species taking center stage. Production and excretion of redox-cycling drugs is an important environmental source of reactive oxygen species. Pyocyanin, a phenazine produced by Pseudomonas [179], plumbagin made by the Plumbago plant [180], and juglone from the black walnut tree [88] are prime examples of nature's taking advantage of redox-cycling compounds to poison competitors. While the organisms that produce these compounds are unaffected by them – or, in the case of phenazines, actually benefit directly [44, 190] – other organisms that come into contact with the compounds take them up, where they react with various components in the cell, such as flavins and components of the respiratory chain, stealing electrons and producing reactive oxygen species [73]. These reactive oxygen species made from the redox-cycling drugs prevent other organisms from inhabiting the same area as the producer and serve as a natural form of biochemical warfare. However, some organisms have responded by evolving specific responses to these redox-cycling drugs. These responses include defenses against oxidative stress, as well as drug modification and efflux systems that render the compounds ineffective [68, 70, 105, 167, 169]. This is just one more example of evolution to survive oxidative stress in response to organisms that use it as a weapon.

While this evolutionary battle with oxidative stress is more recognizable when comparing hosts and pathogens, it is also important to consider how those organisms that produce the reactive oxygen species or redox-cycling drugs avoid poisoning themselves. For example, how do macrophages keep reactive oxygen species from damaging other host cells? In this case, invading microorganisms are engulfed into a specific compartment within the macrophage, the phagosome, where reactive oxygen species are generated. The macrophage isolates the stress within itself, thus preventing other cells from experiencing the negative effects of these oxidants. Another, more striking example of this conundrum is the survival of lactic acid bacteria that produce H_2O_2 [66, 162, 170]. These bacteria can produce millimolar levels of H_2O_2 under some conditions but are still able to survive. While excretion of H_2O_2 provides a competitive advantage to these

organisms, they must, at the same time, protect their own proteins and DNA from damage.

It seems that lactic acid bacteria have adopted a two-pronged approach to surviving these high levels of H_2O_2. First, lactic acid bacteria have cleared their proteome of some of the iron-dependent processes that make other cells vulnerable to H_2O_2 [34, 170]. This does create a need for lactic acid bacteria to acquire some metabolites from their environment, but it also limits the number of processes that are sensitive to oxidation. However, this alone would not be enough, as any amount of iron in the cell at these concentrations of H_2O_2 would certainly lead to DNA damage, if not damage to some proteins as well. Therefore, the second approach taken by lactic acid bacteria has been to evolve a mangano-centric metabolism by importing millimolar levels of manganese, allowing them to become nearly independent of iron [7]. This allows these bacteria to survive the high levels of H_2O_2 they can produce while killing their competition.

1.7.2 Habitation of niche environments

Another important aspect of the ecological importance of oxidative stress has little to do with surviving the onslaught of reactive oxygen species imposed by other organisms, but everything to do with surviving production of oxidants endogenously. While strict anaerobes cannot survive even small amounts of oxygen and strict aerobes cannot survive without it, organisms that have evolved the ability to survive in either environment, or at least to tolerate low levels of oxygen, gain a competitive advantage by expanding the scope of potential habitats. This is what allows some organisms to inhabit the niche environments at the interface of aerobic and anaerobic environments. An example of such an environment is the sediment in most bodies of water or deep in the water column of exceptionally stagnant water bodies [22]. Here, organisms are forced to cope with small amounts of oxygen and therefore the potential for exposure to reactive oxygen species. Organisms that inhabit this interface have the unique ability to benefit from the availability of oxygen for aerobic reactions while also having access to metabolites that are only produced in anaerobic environments. Sulfide-oxidizing bacteria

provide an excellent example of this behavior [53, 94]. Sulfide is oxidized to sulfur in the anoxic layer, since sulfur can be stored in large quantities in the cell. The bacteria then migrate to the aerobic layer where they can use oxygen to further oxidize the sulfur to sulfate. Sulfate-reducing bacteria, which were long thought to be strict anaerobes, have also been observed to take advantage of life at the interface of the oxic/anoxic layers [45, 57, 107, 128, 192]. These organisms have evolved to take full advantage of life at the interface.

1.7.3 Why are there obligate anaerobes?

Throughout evolutionary history, life has sought to combat the deleterious effects of reactive oxygen species associated with an aerobic lifestyle in order to take full advantage of the benefits oxygen can provide. However, there are many examples of organisms that have not made this evolutionary shift. Thus, the puzzle remains as to why still obligate anaerobes remain when there are so many examples of defense and survival mechanisms that can confer resistance to reactive oxygen species.

One early hypothesis as to why obligate anaerobes remain prevalent was that they simply lacked scavenging systems and were unable to defend themselves against reactive oxygen species [123]. This turns out to be completely false. In fact, obligate anaerobes have been demonstrated to use a variety of combinations of scavenging systems including catalases, peroxidases, superoxide dismutases, and superoxide reductases [91, 119, 129, 130, 145]. Another idea was that despite the presence of scavenging systems, anaerobes did not have the ability to induce them. This also turns out to be false, as there are numerous instances of PerR and OxyR homologs in obligate anaerobes, as well as mutations in PerR that confer some degree of aerotolerance [77, 84, 142]. Therefore, there must be some other reason that obligate anaerobes are unable to tolerate aerobic environments.

Another possible explanation for the persistence of obligate anaerobes is that the problem is not reactive oxygen species themselves, but oxygen itself. Indeed, a number of anaerobic reactions involve low potential or free-radical enzymes that are easily

oxidized by molecular oxygen [71, 139]. These processes are therefore non-functional in aerobic habitats and cannot be rescued by any amount of scavenging enzymes.

With these findings taken together, the implication is that abrupt and complete oxygenation may immediately incapacitate obligate anaerobes, preventing them from mounting any kind of response to reactive oxygen species. However, when they are gradually exposed to low levels of oxygen, they may be capable enough of carrying out metabolism to engage their defense mechanisms and persist through the stress. Thus, even strict anaerobes may not be completely intolerant of oxygen but have also evolved to tolerate it in moderation.

1.8 SCOPE OF THIS THESIS

1.8.1 Damage to mononuclear iron enzymes by H_2O_2

When Hpx^- (catalase and peroxidase deficient) cells are stressed with H_2O_2 in minimal glucose medium, only one phenotype is apparent: an auxotrophy for aromatic amino acids. In this study I set out to determine the target of H_2O_2 responsible for this phenotype. In this work I also identified an additional phenotype of H_2O_2 stress in the Hpx^- strain – sensitivity of the pentose phosphate pathway.

1.8.1.1 Ribulose 5-phosphate epimerase

The discovery of a sensitive target in the pentose phosphate pathway led to the discovery that ribulose 5-phosphate epimerase (Rpe) was sensitive to H_2O_2. This finding revealed a unique class of H_2O_2 target as the only proteins previously known to be damaged directly by H_2O_2 were Fe-S cluster dehydratases. The novelty of this discovery was compounded by the fact that Rpe was not thought to be activated by iron [1]. This work showed that Rpe uses iron as its physiological cofactor and that mononuclear iron proteins are a novel class of H_2O_2 sensitive protein.

1.8.1.2 DAHP synthase and the aromatic amino acid auxotrophy

With the finding that mononuclear iron proteins were sensitive to H_2O_2, I was able to identify DAHP synthase, another mononuclear iron protein and the first enzyme in the aromatic amino acid biosynthetic pathway, as the cause of the aromatic amino acid auxotrophy. Work published on the same auxotrophy in SOD mutants identified transketolase as the target of $O_2^{\cdot -}$ [12], but I showed that transketolase is not sensitive to H_2O_2. I also showed that Hpx^- cells attempt to compensate for a lack of DAHP synthase activity by inducing expression of the *aroF* gene, which encodes the tyrosine-responsive isozyme of DAHP synthase, but this does not sufficiently increase activity to allow growth.

1.8.2 Protection of mononuclear iron enzymes by manganese

Manganese has been known to have protective effects during H_2O_2 stress. The second goal of this study was to determine whether manganese could protect mononuclear iron proteins from H_2O_2. In this work, I showed that manganese can protect two mononuclear iron proteins, Rpe and DAHP synthase, during H_2O_2 stress. I also showed that manganese was able to activate these proteins and provide resistance to H_2O_2 in vitro.

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CHAPTER 2: THE IRON ENZYME RIBULOSE-5-PHOSPHATE 3-EPIMERASE IN *ESCHERICHIA COLI* IS RAPIDLY DAMAGED BY HYDROGEN PEROXIDE BUT CAN BE PROTECTED BY MANGANESE[*]

2.1 INTRODUCTION

Catalases and peroxidases were among the first enzymes discovered, and their ubiquity among the biota prompted biologists to speculate that hydrogen peroxide (H_2O_2) must be a common by-product of the aerobic metabolism, and that if not scavenged it must critically harm cells [26]. Since then workers have recognized that H_2O_2 is also generated by extracellular processes, including photochemical processes in natural waters and the autoxidation of sulfur and metal species at interfaces between anoxic and oxic sediments. Perhaps more critically, H_2O_2 is generated by the antimicrobial responses of both plant and mammalian higher organisms as a strategy to stave off infection [12, 28]. The local H_2O_2 concentrations rise to micromolar levels, which is sufficient to suppress microbial growth.

Virtually all microbes engage specialized stress responses to fend off exogenous H_2O_2. Among the bacteria, the OxyR and PerR systems are widespread [4, 24]. OxyR, which has been closely studied in enterics, is a transcription factor that is activated when H_2O_2 oxidizes a key cysteine residue; the subsequent formation of a disulfide bond locks the protein into an active form that stimulates the transcription of *ca.* two dozen genes [5, 43]. Mutants that lack OxyR grow poorly or not at all in environments that contain H_2O_2.

Still, fundamental aspects of H_2O_2 stress remain poorly understood. Perhaps most importantly, the primary intracellular targets of H_2O_2 have not been fully identified. A

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technical difficulty has hindered work on this problem: When laboratory cultures are stressed with micromolar H_2O_2, growth briefly stops, but the H_2O_2 is quickly scavenged and growth resumes. Under these circumstances it has been difficult to identify the specific cellular processes and biomolecules that are affected. One way around this problem has been the use of strains that lack scavenging enzymes [34]. *E. coli* catalase/peroxidase mutants (*katG katE ahpCF*, denoted Hpx^-) grow at wild-type rates in anaerobic cultures. When inoculated into aerobic media, H_2O_2 accumulates to *ca*. 1 micromolar, which equilibrates between the cytoplasm and the extracellular environment. This concentration exceeds the threshold that activates OxyR, and indeed Hpx^- *ΔoxyR* mutants die quickly in aerobic media [30]. Thus the Hpx^- strains offer the opportunity to identify the primary biomolecules that H_2O_2 damages, as well as the key defensive strategies that OxyR triggers.

Thus far it has become clear that H_2O_2 damages DNA through standard Fenton chemistry. The iron that is involved belongs to the cellular pool of unincorporated iron, some of which presumably associates with nucleic acids [31]. A ferryl radical (FeO^{2+}) is the immediate product, but ultimately a hydroxyl radical is released that can directly attack both the sugar and base moieties of DNA [17]. Intracellular H_2O_2 also disables a family of dehydratases that contain [4Fe-4S] clusters; the chemistry is similar, in that H_2O_2 directly oxidizes a catalytic iron atom that is exposed to solvent [18]. The oxidized cluster is unstable, the key iron atom dissociates, and enzyme activity is lost. These enzymes were first shown to be oxidative targets in studies of superoxide [9-11, 23, 25]. Exacerbating this situation, H_2O_2 also inhibits the cellular iron-sulfur-cluster assembly machinery, likely by oxidizing nascent clusters as they are assembled on the IscU scaffold protein [19]. Finally, H_2O_2 has been shown to disrupt the Fur iron-sensing system [40]. In this case the proximate cause has not been demonstrated, but it seems likely that H_2O_2 oxidizes the Fe^{2+} iron atom that normally binds to the Fur transcription factor.

The OxyR regulon stimulates the synthesis of several proteins whose roles are well-suited to addressing these effects. The HPI catalase and AhpCF peroxidase are induced to drive back down the intracellular H_2O_2 level [43]. The Dps iron-storage

protein sequesters unincorporated iron and thereby diminishes the amount of DNA damage [13, 15, 30, 41]. An alternative iron-sulfur assembly machine, the Suf system, is resistant to H_2O_2 and is induced to compensate for the failure of the Isc system [19]. The Fur regulator is induced to higher levels as a hedge against its lower efficiency [40, 42].

Recently it was discovered that the induction of a manganese import system was also critical, as Hpx$^-$ *ΔmntH* mutants stop growing when they are aerated [2]. *E. coli* normally does not import significant amount of manganese. I speculated that *E. coli* might employ ferrous iron as a cofactor in non-redox enzymes, which would place such enzymes at risk during periods of H_2O_2 stress. Induction of the MntH transporter might enable manganese to replace iron in these enzymes; since manganese does not react with H_2O_2, the enzymes might be protected.

In the present study I sought to identify cellular processes that are inhibited by micromolar H_2O_2. I identified the pentose-phosphate pathway as one such target and determined that the bottleneck arises because of damage to a mononuclear enzyme that contains ferrous iron. When manganese is provided to these cells, the enzyme is protected and the pathway function is restored.

2.2 RESULTS

2.2.1 Ribulose-5-phosphate 3-epimerase is vulnerable to hydrogen peroxide damage in vivo

Strains of *E. coli* that cannot scavenge H_2O_2 gradually accumulate *ca*. 1 micromolar H_2O_2 [34]. This level moderately exceeds the dose that activates that OxyR response, and so it is likely that these strains confront protracted H_2O_2 stress of a magnitude similar to that found in some natural habitats. Growth studies of this strain have shown that most catabolic and biosynthetic pathways continue to function, which conforms with the observation that this level of H_2O_2 is innocuous to the vast majority of enzymes. However, in the process of testing the H_2O_2 sensitivity of central metabolic

pathways, I noted that an Hpx$^-$ Δ*edd* mutant is unable to grow in aerobic gluconate medium (Figure 2.1A). This strain is designed to require flux through the pentose-phosphate pathway (Figure 2.2). The cells began to lag when the H_2O_2 concentration had reached approximately 0.8 μM (Figure 2.3), indicating that very low levels of H_2O_2 can inactivate this pathway.

The fact that Hpx$^-$ *edd*$^+$ cells can grow on gluconate via the alternative Entner-Doudoroff pathway implied that the block must occur downstream of gluconate import. The five pentose-phosphate-pathway enzymes were assayed in extracts that had been prepared from aerobic cultures of wild-type and Hpx$^-$ strains. No differences were found in the levels of 6-phosphogluconate dehydrogenase (Gnd), ribose-5-phosphate isomerase (Rpi), transketolase (Tkt), and transaldolase (Tal) (Figure 2.1B). However, ribulose 5-phosphate 3-epimerase (Rpe) showed low activity. In order to determine whether H_2O_2 can inactivate Rpe directly, Hpx$^-$ cells were grown anaerobically, cell extracts were prepared, and the extracts were then briefly challenged with H_2O_2 in vitro. Rpe rapidly lost activity (Figure 2.1C). Even at 0^o C, 5 μM H_2O_2 inactivated most of the enzyme within 1 minute (Figure 2.1D); the measured rate constant for inactivation (4000 M^{-1} s^{-1}) approximated those of H_2O_2-sensitive Fe/S dehydratases [18].

2.2.2 Rpe can be metallated by various divalent metals which result in variable properties

In order to study Rpe in vitro, Rpe from *E. coli* was purified using a His tag, which was removed after purification. Rpe is known to require a divalent metal for activity, but the identity of the metal used in vivo remained uncertain [1]. A previous study of Rpe from *S. pyogenes* reported that it co-purified with substantial Zn^{2+}, leading the authors to speculate that this is its physiological metal. Akana *et al.* reported that Mn^{2+} and Co^{2+} would also activate Rpe in vitro, but that Mg^{2+} and Fe^{2+} would not. However, these experiments were conducted under aerobic conditions, and the Fe^{2+} likely oxidized to Fe^{3+}. I used chelators to remove any associated metals from the purified Rpe from *E. coli*, and I then tested various metals for their ability to activate the enzyme in vitro under anaerobic conditions. Of the nine metals used, Ca^{2+}, Cd^{2+}, Cu^{2+}, Mg^{2+}, and

Ni^{2+} did not activate Rpe above background levels. However, Co^{2+}, Fe^{2+}, Mn^{2+}, and Zn^{2+} were able to activate Rpe in vitro.

The kinetic constants of the four metalloforms of Rpe were determined (Table 2.1). Strikingly, the Zn^{2+} metalloform had a much lower k_{cat} and higher K_M than the other metalloforms; thus the catalytic efficiency (k_{cat}/ K_M) of the Zn^{2+} metalloform was much worse than those of the Mn^{2+}, Fe^{2+}, and Co^{2+} forms, which were roughly comparable.

The dissociation rates for each of the activating metals were also measured (Table 2.1). Despite providing the most catalytically efficient metalloform, Mn^{2+} rapidly dissociated from Rpe, while the Fe^{2+} metalloform was moderately more stable. Interestingly, Zn^{2+} bound tightly to the enzyme.

Of these four metals, only Fe^{2+} and Zn^{2+} are found in *E. coli* in substantial concentrations under normal growth conditions. *E. coli* lacks a Co^{2+} importer, and Mn^{2+} is not actively imported during routine growth [2]. It seemed likely that Fe^{2+} is the metal that provides activity to Rpe under normal physiological conditions.

2.2.3 Fenton chemistry is responsible for the inactivation of Rpe

The proposed mechanism of Rpe implies that the metal will be exposed to solutes, including H_2O_2 (Figure 2.4); therefore, I tested whether Fenton chemistry was responsible for loss of Rpe activity. The sensitivity to H_2O_2 of each of the metalloforms of Rpe was tested. When purified Rpe was loaded with Mn^{2+}, Co^{2+}, or Zn^{2+}, there was no loss in activity when challenged with H_2O_2 (Figure 2.5). However, when Rpe was metallated with Fe^{2+}, the activity was quickly lost. Since the enzyme that is found in cell extracts is acutely sensitive to H_2O_2, I concluded that Fe^{2+} is indeed the native cofactor that provides activity in lab cultures.

Reactions between H_2O_2 and Fe^{2+} initially generate ferryl radicals, which have a substantial lifetime before decomposing into Fe^{3+} and a hydroxyl radical [16, 33]. Hydroxyl radicals react at near-diffusion-limited rates with most biomolecules, including proteins, raising the prospect that H_2O_2 exposure might lead to covalent Rpe damage. I

found, however, that when iron-loaded Rpe was inactivated with H_2O_2, about 80% of the initial Rpe activity could be recovered by adding Fe^{2+} back to the enzyme (Figure 2.6A). Reductants alone were insufficient, confirming that the oxidized iron atom had dissociated from the enzyme. Similar results were obtained by the addition of Co^{2+} or Mn^{2+}. Full activity was never recovered. Thus I concluded that at most only a very minor fraction of the protein was critically and irreversibly damaged; instead, most of the activity loss was due to the dissociation of iron.

In vivo experiments showed a similar outcome. When Hpx^- Δ*edd* cells were exposed to a bolus of H_2O_2, activity quickly declined. After the H_2O_2 was removed from the culture, activity rebounded to about 70% of the original activity, even though new protein synthesis was blocked (Figure 2.6B). The recovery of activity inside the cell reached the same level as when activating metals were added back to the lysate of the H_2O_2-treated cells.

2.2.4 Repeated rounds of inactivation are required to permanently damage Rpe

The data suggested that when iron-loaded Rpe is oxidized by H_2O_2 the catalytic iron cofactor is oxidized and lost; however, despite the presumptive formation of a hydroxyl radical, only a small fraction of those events caused irreversible protein damage. Inactivation was predominantly due to metal loss. In vivo, however, continual enzyme re-metallation should occur. When Rpe activity was tracked during extended Hpx^- growth in aerobic medium, activity quickly fell. At early time points most of the enzyme was in a non-metallated but reactivatable form, as activity could be recovered by metal addition in vitro. However, the amount of recoverable activity progressively declined, suggesting that Rpe gradually accrued irreversible damage from multiple rounds of Fenton reactions (Figure 2.6C).

In order to replicate cycles of damage and re-metallation in vitro, purified Rpe was exposed to iron, ascorbate, and oxygen in vitro. The oxidation of Fe^{2+} by oxygen ensured the constant presence of a low level of H_2O_2 [29], while the reduction of iron by ascorbate regenerated Fe^{2+}. At time points the iron and H_2O_2 were removed by DTPA

and catalase, and the amount of functional Rpe polypeptide was determined by the addition of Co^{2+} prior to assay. When ascorbate was omitted, activity diminished only at first, and it thereafter remained constant at about 65% of the starting activity, consistent with a single cycle of enzyme damage. However, when ascorbate was added to enable the cyclical activation and oxidation of the enzyme, activity dropped steadily, down to 15% after one hour and about 3% after 2 hours (Figure 2.6D). Taken together, these results indicate that a single bolus of Fenton chemistry irreversibly damages only a fraction of the Rpe molecules. Complete inactivation of the population results from repeated cycles of the reaction.

2.2.5 Manganese protects Rpe from damage by hydrogen peroxide

The induction of a manganese importer, MntH, has been shown to be a critical aspect of the response of *E. coli* to H_2O_2 [2, 20]. It was proposed recently that the role of Mn in the protection against H_2O_2 stress might be to replace Fe^{2+} in the mononuclear metal sites of enzymes, thereby diminishing the frequency of Fenton chemistry in those sites. However, no specific examples of this hypothesis had been presented. To test this idea with respect to Rpe, Hpx^- Δ*edd* cells were grown aerobically on gluconate with or without Mn supplementation. As shown in Figure 2.7A, Mn addition fully suppressed the aerobic growth phenotype in Hpx^- Δ*edd* cells. To verify that the protective effect was due to protection of Rpe, its activity was measured in samples harvested from cultures grown both with and without Mn supplements. Figure 2.7B shows that the supplements boosted Rpe activity by several-fold at the ten-hour time point, when unsupplemented cells began to lag. Because the dissociation rate of Mn in vitro is so fast, these assays almost certainly underrepresented the activity of Rpe containing Mn. Therefore, I sought to quantify the amount of functional Rpe polypeptide by providing Co^{2+} to the extracts. The samples from Mn-supplemented cultures showed nearly 100% activity, compared to less than 25% activity from untreated cultures (Figure 2.7B). These data support the idea that Mn protects Rpe from H_2O_2 by replacing Fe^{2+} in the active site during H_2O_2 stress.

2.2.6 Iron must be accessible to solvent in order for enzymes to be vulnerable to hydrogen peroxide

The full range of enzymes that use Fe^{2+} as a cofactor in vivo—and therefore that might be vulnerable to H_2O_2—is unknown. Presumably many enzymes that require divalent metal cofactors might be able to use Fe^{2+}. However, Fenton chemistry requires the direct binding of iron to H_2O_2, and so I suspected that only those enzymes that might have under-coordinated iron in their active sites are potentially vulnerable. To test this idea, transketolase was examined. Transketolase requires a divalent metal to form a bridge between the polypeptide and a thiamine pyrophosphate (TPP) cofactor, but the metal does not participate in the reaction chemistry. The metal itself is buried beneath the bound cofactor and is not exposed to solvent [Isupov *et. al*, PDB code 1QGD]. I determined that Fe^{2+} was able to activate transketolase as efficiently as did Mg^{2+}. However, in contrast to Rpe, the addition of H_2O_2 did not affect the activity of the Fe^{2+}/TPP-loaded enzyme (Figure 2.8). These results demonstrate that enzymes that use Fe^{2+} as a metal cofactor are sensitive to H_2O_2 only if the metal site is exposed to solvent; if the Fe^{2+} is fully coordinated, Fenton chemistry is blocked and the enzyme is not vulnerable to H_2O_2. This feature presumably will circumvent damage to many enzymes, like transketolase, that might occasionally or frequently bind Fe^{2+} as a divalent cofactor.

2.3 DISCUSSION

In addition to their facility at redox reactions, transition metals excel at surface chemistry, in which they directly coordinate substrates and thereby activate them for a wide variety of reaction types. Rpe is an example of the latter: its mononuclear metal atom binds the carbonyl and carboxylate moieties of ribulose-5-phosphate, facilitating epimerization by stabilizing the deprotonated anionic intermediate. In addition, over the course of the reaction the metal alternatively coordinates and releases the aspartyl residues that act as proton acceptors and donors to the substrate. Although a non-transition metal such as magnesium can suffice in enzymes where its sole role is to

neutralize charge, transition metals are often requisite when the metal environment must change during the reaction cycle, as they can tolerate changes in their coordination sphere with minimal activation energy.

This study indicates that iron is the transition metal that activates Rpe. This makes both ecological and evolutionary sense, as iron is typically abundant in anaerobic habitats—including both the mammalian gut in which *E. coli* dwells and the anaerobic world in which Rpe originally evolved. Indeed, the service of ferrous iron in non-redox enzymes is probably systematically under-recognized, because iron is quickly oxidized and released when these enzymes are studied in aerobic buffers. Thus, whereas in vitro experiments suggest that manganese can activate scores of enzymes in *E. coli* [http://www.Ecocyc.org/], in reality manganese is scarcely imported under routine growth conditions [2]. More likely, iron is the cognate metal of many of these enzymes.

2.3.1 Fe enzymes are targets of oxidants

Catalase and peroxidase activities are sufficient to cope with the H_2O_2 that is formed as a by-product of aerobic metabolism, but the diffusion of H_2O_2 into the cell from environmental sources can nevertheless drive its steady-state levels above the threshold of toxicity [35]. Rpe, and probably mononuclear iron enzymes in general, now join iron-sulfur dehydratases as primary classes of enzymes that this H_2O_2 damages. In both cases H_2O_2 directly oxidizes a solvent-exposed iron atom that normally serves to bind substrate; the oxidized iron atom then dissociates from the enzyme. Interestingly, the hydroxyl-like oxidants that are formed need not fatally damage the active sites of either type of enzyme—the enzymes can be repeatedly reactivated, both in vivo and in vitro. When H_2O_2 oxidizes iron-sulfur clusters, hydroxyl radical release is averted when the nascent ferryl radical abstracts a second electron from the cluster [18]. I speculate that Rpe might similarly be spared covalent damage because the ferryl radical usually dissociates from the active site and does not release a hydroxyl radical until it enters the bulk solution. Upon reflection, it is probably an essential feature of iron-containing enzymes that they are not irreversibly damaged upon each encounter with H_2O_2. Even in innocuous habitats wild-type *E. coli* contains about 20 nM steady-state H_2O_2 from

endogenous sources [35]; because the inactivation rates of these enzymes are likely 10^4-10^5 M^{-1} s^{-1} at physiological temperatures (Figure 2.1D) [12], they are likely to be oxidized by H_2O_2 on the order of every 10 min. This situation would be intolerable were the polypeptide irreparably damaged each time.

2.3.2 The role of alternative metals in Fe enzymes

The reliance of metabolism upon iron-cofactored enzymes presents problems during periods of either iron starvation or oxidative stress. One apparent solution is to replace iron with manganese. When the problem is iron scarcity, the deactivation of the Fur repressor stimulates synthesis of MntH, the manganese importer [20]. Presumably the resultant manganese influx allows mononuclear enzymes such as Rpe to continue to work. Because manganese does not share the reduction potential of iron, the solution is slightly more complicated for redox enzymes: key iron-dependent enzymes, such as superoxide dismutase and ribonucleotide reductase, are replaced by distinct isozymes that bind and poise manganese at an appropriate potential [6, 27].

The induction of the manganese transporter is not sufficient to protect these enzymes from H_2O_2, however, if the H_2O_2-stressed cell still contains ample iron that might yet out-compete manganese for enzyme binding. Thus the induction of MntH by OxyR is supplemented by its induction of the Fur repressor [42], which inhibits expression of iron importers, and of Dps, which sequesters intracellular iron in a ferritin-like storage protein [13, 15, 30, 41]. In collaboration these systems ensure that the iron/manganese ratio tips towards the latter.

I was unable to directly demonstrate that the Rpe proteins recovered from H_2O_2-stressed cells contain manganese; by the point of assay, manganese was not bound. This is the expected outcome of the rapid dissociation of this weakly bound metal during extract preparation. However, it is clear that manganese import blocked enzyme damage and sustained activity. An alternative model for the anti-oxidant effects of manganese [7, 8, 22, 37-39] has been that it acts as a chemical scavenger of reactive oxygen species, including H_2O_2 [14, 21, 22, 32, 37]. That model seems not to apply to *E. coli*, as direct

measurements showed that manganese import did not affect H_2O_2 levels [2]. Further, I found that excess cobalt, which can also activate Rpe but does not chemically degrade H_2O_2, was able to substitute for manganese in preserving the function of the pentose-phosphate pathway during H_2O_2 stress (Figure 2.9). It seems possible that this manganese protection scheme operates constitutively in those lactic acid bacteria that produce H_2O_2 in high quantities, as these bacteria characteristically require millimolar levels of intracellular manganese for good growth [3]. Work in the Daly lab has suggested that additional mechanisms of manganese-based protection might also occur in these organisms [39]. It will be interesting to see whether these organisms have evolved enzymes that are better at binding manganese than is *E. coli* Rpe, or if the requirement for such high intracellular manganese is a reflection of weak binding in these cells as well.

It has long been of interest to identify the mechanisms that ensure that enzymes bind their cognate metals. The example of *E. coli* experiencing H_2O_2 stress, during which cellular Mn^{2+} rises from 15 to 180 μM [2], indicates that metallation is substantially controlled by the relative concentrations of the competing metals. But this example also points out that this level of control is not enough, since zinc, which binds more tightly to the Rpe active site than does either manganese or iron, is abundant inside the cell. Indeed, when manganese-loaded Rpe was co-incubated with zinc in vitro, activity steadily declined as the association/dissociation equilibrium of manganese was interrupted by the permanent binding of zinc (Figure 2.10). The quick solvation of manganese and iron from mononuclear enzymes may be the counterpoint to their facility at the exchange reactions which allow them to be good surface catalysts. Inside the cell competition from zinc is presumably suppressed by other intracellular ligands, such as the glutathione pool, that substantially sequester it. Whether the cell also employs systems that either actively load iron/manganese into mononuclear enzymes like Rpe, or that facilitate the dissociation of inappropriately bound metals, remains to be determined.

2.4 EXPERIMENTAL PROCEDURES

2.4.1 Reagents

Amino acids, antibiotics, ascorbic acid, catalase (from bovine liver), cobalt (II) chloride hexahydrate, diethylenetriaminepentaacetic acid (DTPA), ethylenediaminetetraacetic acid(EDTA), D-erythrose 4-phosphate sodium salt, ferrous ammonium sulfate hexahydrate, fructose 6-phosphate dipotassium salt, a-glycerophosphate-triosephosphate isomerase from rabbit muscle, imidazole, manganese (II) chloride tetrahydrate, β-nicotinamide adenine dinucleotide reduced disodium salt (NADH), β-nicotinamide adenine dinucleotide phosphate sodium salt (NADP), 6-phosphogluconic acid tri(cyclohexylammonium) salt, D-ribose 5-phosphate disodium salt, D-ribulose 5-phosphate disodium salt, transketolase (from yeast), D-xylulose 5-phosphate sodium salt, zinc chloride, and 30% H_2O_2 were from Sigma. Glycylglycine was from Acros Organics, and Tris-HCl was from Fisher.

2.4.2 Bacterial growth

Luria-Bertani (LB) medium contained 10 g tryptone, 10 g NaCl, and 5 g yeast extract per liter. Minimal medium was composed of minimal A salts supplemented with 0.5 µg/mL thiamin and 0.5 mM of the following amino acids: histidine, phenylalanine, tryptophan, and tyrosine. Carbon sources glucose or gluconate were added at 0.2%, where indicated.

Anoxic growth was carried out in a Coy anaerobic chamber which contained an atmosphere consisting of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. Cultures were maintained at 37°C. Aerobic cultures were grown by shaking cultures vigorously at 37°C.

2.4.3 Growth curves

Cells were first grown overnight to stationary phase in anaerobic minimal glucose medium. Cultures were then inoculated to ~ 0.01 OD and grown anaerobically to 0.2 OD. Cells were then washed twice with minimal A salts, and used to inoculate fresh aerobic minimal gluconate medium to 0.005 OD. Where indicated, 50 µM $MnCl_2$ was added both to anaerobic precultures and to aerobic growth medium.

2.4.4 Enzyme assays

2.4.4.1 In vivo exposure

Cultures of JI418 and JI422 cells used for in vivo exposure assays were grown anaerobically in minimal glucose medium at least four generations to an OD of 0.4. A 25 mL aliquot was removed to determine initial activity, while 50 mL of the remaining culture was washed twice in 25 mL minimal A salts. The washed cells were used to inoculate 100 mL minimal A gluconate medium to 0.05 OD. Cultures were grown shaking at 37°C to an OD of 0.1. Next, 50 mL cultures were moved into the anaerobic chamber and washed twice in ice-cold 25 mL 50 mM glycylglycine buffer, pH 8.5, and resuspended in 1 mL (2 mL for anaerobic sample) 50 mM glycylglycine buffer, pH 8.5 containing 1 mM DTPA. Samples were then sonicated for 2 minutes (3 seconds on/3 seconds off) and lysates were cleared by centrifugation at 14000 x g for 3 minutes. Samples were then assayed immediately as described below.

2.4.4.2 In vitro exposure

Cultures of LC106 cells used for in vitro H_2O_2 exposure assays were grown anaerobically in minimal glucose medium at least four generations to an OD of 0.4. All extract preparation was done in an anaerobic chamber. The 25 mL cultures were washed twice in ice-cold 25 mL 50 mM glycylglycine buffer, pH 8.5, and resuspended in 1 mL 50 mM glycylglycine buffer, pH 8.5 containing 1 mM DTPA. Samples were then sonicated for 2 minutes (3 seconds on/3 seconds off) and lysates were cleared by centrifugation at 14000 x g for 3 minutes. Samples were then assayed immediately as

described below. For H_2O_2 treatment, an aliquot was diluted 1:10 in buffer containing 10 µM H_2O_2 and incubated for 2 minutes. This mixture was then diluted in half with buffer containing a 1:10000 dilution of catalase. Samples were then assayed as described below.

2.4.4.3 6-phosphogluconate dehydrogenase

6-phosphogluconate dehydrogenase was assayed by adding extract at a final dilution factor of 1:50 to a reaction mixture containing 20 mM Tris-HCl buffer, pH 7.8, 6.9 mM $MgCl_2$, 4.2 mM 6-phosphogluconate, and 0.2 mM NADP in a final reaction volume of 500 µL in a sealed, anaerobic cuvette. Absorbance was measured at 340 nm.

2.4.4.4 Transaldolase

Transaldolase was assayed by adding extract at a final dilution factor of 1:50 to a reaction mixture containing 50 mM glycylglycine buffer, pH 8.5, 5 mM DTPA, 1 unit of α-glycerophosphate dehydrogenase and 10 units of triosephosphate isomerase, (obtained as a pre-mixed suspension) 2.8 mM fructose 6-phosphate, 0.2 mM erythrose 4-phosphate, and 0.2 mM NADH in a final reaction volume of 500 µL in a sealed, anaerobic cuvette. Absorbance was measured at 340 nm.

2.4.4.5 Transketolase

Assay for transketolase was assayed by adding extract at a final dilution factor of 1:50 to a reaction mixture containing 50 mM glycylglycine buffer, pH 8.5, 5 mM DTPA, 1 unit of a-glycerophosphate dehydrogenase and 10 units of triosephosphate isomerase, (obtained as a pre-mixed suspension) 1 mM xylulose 5-phosphate, 1 mM ribose 5-phosphate, and 0.2 mM NADH in a final reaction volume of 500 µL in a sealed, anaerobic cuvette. Absorbance was measured at 340 nm.

2.4.4.6 Ribose 5-phosphate isomerase

Assay for ribose 5-phosphate isomerase was identical to the assay for transketolase, except that 1 mM ribulose 5-phosphate was added instead of ribose 5-

phosphate. The mixture also contained 1 unit of transketolase from a stock of 5 units/mL that was pre-treated with 0.2 mM $MgCl_2$ and 2 mM thiamin pyrophosphate.

2.4.4.7 Ribulose 5-phosphate epimerase

Assay for ribulose 5-phosphate epimerase was identical to the assay for transketolse, except that 1 mM ribulose 5-phosphate was added instead of xylulose 5-phosphate. The mixture also contained 1 unit of transketolase from a stock of 5 units/mL that was pre-treated with 0.2 mM $MgCl_2$ and 2 mM thiamin pyrophosphate.

2.4.5 Purification of Rpe

Rpe was purified by cloning of the *E. coli* Rpe gene into the pET21b vector using the forward primer 5'-GCAATGCATATGAAACAGTATTTGATTGCCCCCTCAATTCTGTCG-3', which included an NdeI restriction site, and the reverse primer 5'-GCAATGCTCGAGTTATTCATGACTTACCTTTGCCAGTTCACTGCG-3', which included an XhoI restriction site. After ligation, plasmids were transformed into BL21 and selected on LB plates containing 50 µg/mL ampicillin. One transformant found to have the correct Rpe sequence was chosen for Rpe purification. *E. coli* strain BL21 containing plasmid pRpe-His10 was grown in LB glucose medium containing 50 µg/mL ampicillin to stationary phase, diluted into fresh LB glucose containing 50 µg/mL ampicillin to 0.006 OD, and grown at 37°C to 0.2 OD. This preculture was then used to inoculate 1 L fresh LB glucose containing 50 µg/mL ampicillin to 0.005 OD and cells were grown at 37°C to 0.6 OD. IPTG was then added to a final concentration of 0.4 mM, the culture was shifted to 15°C, and incubated for 18 hours.

Purification was performed as described by Akana *et al.* with some modifications. Buffers used were: binding buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, and 5 mM imidazole), elution buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, and 1 M imidazole), wash buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, and 60 mM imidazole), and strip buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, and 100 mM EDTA). Cells were centrifuged and washed with 30 mL binding buffer, resuspended in 20 mL binding

buffer, and sonicated. Cell extracts were cleared by centrifugation for 15 minutes at 4000 x g at 4°C. Two His Gravitrap columns (GE Healthcare) were equilibrated at 4°C using 10 mL binding buffer. Each column was loaded with 10 mL cleared extract, washed with a 10 mL mixture of 15% elution buffer and 85% wash buffer. A second wash using 3 mL of a 50% wash buffer and 50% strip buffer mixture was run before elution was conducted by adding 3 mL elution buffer to each column. Eluates were pooled and dialyzed in 3.5 L 50 mM Tris-HCl, pH 8.0 containing 100 mM NaCl and 5 mM $CaCl_2$ at 4°C for 6 hours. Buffer was changed and dialysis was run an additional 6 hours.

To remove the 10-His tag, dialysate was cleaved using a Factor Xa Cleavage/Capture Kit (Novagen). The cleavage mixture contained 800 µL 10X Cleavage/Capture buffer, 100 µL Factor Xa (2U/µL), 6 mL dialysate, and 1.1 mL dH_2O. This mixture was incubated at room temperature for 36 hours, then moved to ice. Factor Xa was then removed according to kit instructions. Flowthrough from multiple columns were pooled together. Next, to remove uncleaved Rpe-His10, a His Gravitrap column was equilibrated with 10 mL 50mM Tris-HCl, pH 8.0 containing 500 mM NaCl at 4°C. Flowthrough from Factor Xa removal was then loaded onto the column and washed with 6 mL 50mM Tris-HCl, pH 8.0 containing 500 mM NaCl. Rpe was mostly retained on the column, so 6 mL binding buffer was used to wash the column, followed by elution of cleaved Rpe using 6 mL of a mixture containing 50% strip buffer and 50% wash buffer. The resulting purified Rpe had a protein concentration of 1.52 mg/mL.

2.4.6 Determination of K_M and k_{cat}

Purified Rpe was diluted 1:1000 into 50 mM glycylglycine buffer, pH 8.5, for Co, Fe, and Mn, or 1:100 for Zn in the anaerobic chamber. Rpe was further diluted 100-fold into 50 mM glycylglycine buffer (pH 8.5), with a final concentration of 500 µM metal and incubated for 10 minutes at 23°C. Rpe was then assayed as above using various RuMP concentrations. K_M *and* k_{cat} values were determined by using a double reciprocal plot.

2.4.7 Metal dissociation rates

In the anaerobic chamber, purified Rpe was diluted 1:100 into 50 mM glycylglycine buffer, pH 8.5 containing 500 µM of each metal for 5 minutes before being diluted 10-fold into 50 mM glycylglycine buffer, pH 8.5 containing 1 mM DTPA and incubated at 0°C or 23°C. Aliquots were taken at regular intervals and Rpe activity was measured as described above.

2.4.8 H_2O_2 challenge in vitro

Pure Rpe was diluted 1:10000 (1:1000 for Zn) into 50 mM glycylglycine buffer, pH 8.5 containing 100 µM of each metal for 5 minutes anaerobically before being diluted 10-fold into the Rpe assay mixture. The reaction was run for 6 minutes to determine initial activity before being treated with 5 µL 50 mM H_2O_2 (final concentration of 500 µM). Rates were compared before and after H_2O_2 treatment.

2.4.9 H_2O_2 challenge in vivo

Cultures of LC106 cells used for in vitro H_2O_2 exposure assays were grown anaerobically in minimal glucose medium at least four generations to an OD of 0.4. Chloramphenicol was added to a final concentration of 150 µg/mL and cultures were incubated at 37°C for an additional 5 minutes. A 25 mL aliquot was removed to determine initial activity, while 75 mL of the remaining culture was treated with 100 µM H_2O_2 and shifter to aerobic conditions where the culture was shaken at 37°C for 10 minutes. Catalase was then added and cultures were shifted back to the anaerobic chamber. All extract preparation was done in an anaerobic chamber as described earlier.

2.4.10 Ascorbate cycling in vitro

Pure Rpe was diluted 1:50000 into 50 mM glycylglycine buffer, pH 8.5, containing 20 µM DTPA. $CoCl_2$ was added last to a final concentration of 50 µM, samples were incubated for 10 minutes anaerobically, then assayed for Rpe activity to

determine maximum activity level. For cycling samples, 10 µL pure Rpe was added to 305 µL 50 mM glycylglycine buffer, pH 8.5, and 10 µL of 1 mM DTPA in each of 3 tubes. Next, 25 µL of 1 mM $CoCl_2$ was added to tube 3 and incubated for 10 minutes. Buffer was added to bring the volume of each of the three tubes to 425 µL. To tube 1, 50 µL more buffer was added, while 50 µL 100 mM ascorbate was added to tubes 2 and 3. Finally, 25 µL of 1 mM Fe was added to each of the three tubes. (Final concentrations are: 1:50000 dilution of Rpe, 20 µM DTPA, 50 µM Fe or Co, and 10 mM ascorbate.) Samples were then moved to aerobic conditions and incubated at room temperature. Aliquots of 100 µL were removed periodically and diluted into a final volume of 200 µL containing, in order of addition, 100 µM DTPA, 1:10000 dilution of catalase, and 200 µM $CoCl_2$. Samples were moved back into the anaerobic chamber and assayed for Rpe activity after 10 minutes.

2.4.11 Transketolase challenges

Pure transketolase (Sigma) was metallated by adding 200 µM Fe or $MgCl_2$ to a 50 ug/mL transketolase solution in 50 mM glycylglycine buffer, pH 8.5. After 2 minutes, TPP was added to a concentration of 2 mM either 2 minutes before or after addition of either H_2O_2 or DTPA (final concentration of 500 µM). Samples were then incubated for an additional 2 minutes before being assayed as described above.

2.4.12 Mn protection assays

JI422 cells were grown anaerobically at least four generations to 0.3 OD in minimal glucose medium with or without 50 µM $MnCl_2$. A 25 mL aliquot was removed to measure initial Rpe activity, while remaining 75 mL cultures were washed twice with minimal A salts. Washed cells were then used to inoculate 250 mL aerobic cultures in minimal gluconate medium with or without 50 µM $MnCl_2$ to 0.04 OD. Cultures were grown shaking at 37°C and 50 mL aliquots were removed every 2.5 hours. Extracts were prepared anaerobically as described above. For reactivation, 100 µL of a 1:10 extract dilution was added to 100 µL buffer containing 500 µM $CoCl_2$ to reactivate apo-Rpe. After 10 minutes, Rpe activity was measured as described previously.

2.4.13 H_2O_2 accumulation

Cells were first grown overnight to stationary phase in anaerobic minimal glucose medium. Cultures were then inoculated to ~ 0.01 OD and grown anaerobically to 0.2 OD. Cells were then washed twice with minimal A salts, and used to inoculate fresh aerobic minimal gluconate medium to 0.005 OD. Aliquots were removed periodically and filter sterilized before being frozen on dry ice. After all samples were collected, H_2O_2 concentrations were measured by the Amplex Red/horseradish peroxidase method. Fluorescence was measured using a Shimadzu RF Mini-150 fluorometer.

2.4.14 Inactivation rate determination

Cultures of LC106 cells used for in vitro H_2O_2 exposure assays were grown anaerobically in minimal glucose medium at least four generations to an OD of 0.4. All extract preparation was done in an anaerobic chamber. The 25 mL cultures were washed twice in ice-cold 25 mL 50 mM glycylglycine buffer, pH 8.5, and resuspended in 1 mL 50 mM glycylglycine buffer, pH 8.5 containing 1 mM DTPA. Samples were then sonicated for 2 minutes (3 seconds on/3 seconds off) and lysates were cleared by centrifugation at 14000 x g for 3 minutes. Samples were then diluted 1:10 in buffer containing 1, 5, or 10 µM H_2O_2 and incubated on ice. Aliquots of 90 µL were taken at regular intervals and added to 10 µL of a 1:100 dilution of catalase. Rpe activity was then assayed as described previously.

2.4.15 Substrate protection assay

Purified Rpe was diluted 1:10000 into 50 mM glycylglycine buffer, pH 8.5 containing 100 µM Fe. After 5 minutes, metallated Rpe was then assayed after 1:10 dilution into assay mixture containing either 1 mM or 2 mM ribulose After 5 minutes, metallated Rpe was then assayed after 1:10 dilution into assay mixture containing either 1 mM or 2 mM ribulose. The reaction was run for 3 minutes to determine initial activity before being treated with 5 µL 50 mM H_2O_2 (final concentration of 500 µM). Rates were compared before and after H_2O_2 treatment.

2.5 TABLES

Metal	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($M^{-1} \cdot s^{-1}$)	$t_{1/2}$ of metal dissociation (23°C)
Fe^{2+}	67000 +/- 7700	1.6 +/- 0.2	4.2×10^7	50 min
Mn^{2+}	30400 +/- 1700	1.8 +/- 0.1	1.7×10^7	3.5 min
Co^{2+}	14700 +/- 900	2.4 +/- 0.4	0.6×10^7	20 hours
Zn^{2+}	1300 +/- 100	4.8 +/- 0.5	0.02×10^7	8 hours

Table 2.1: Properties of various Rpe metalloforms. k_{cat} and K_M values were determined as described in Materials and Methods. Error represents standard deviation from the mean of three independent measurements. K_{cat}/K_M values were derived using the average values for k_{cat} and K_M. Dissociation rates were determined by measuring Rpe activity of aliquots taken over time from a mixture containing metallated Rpe diluted into an excess of DTPA and incubated at the temperature specified. Measurements represent the averages of three independent experiments. ND is not determined.

Strain	Genotype	Reference
MG1655	F^- wild type	*E. coli* Genetic Stock Center
LC106	Δ*ahpF*::*kan* Δ(*katG*::Tn*10*)1 Δ(*katE12*::Tn*10*)	[36]
JI418	Δ(*edd-zwf*)22~zeb-1::Tn*10*	Laboratory Stock
JI422	Δ(*edd-zwf*)22~*zeb*-1::Tn*10* Δ*ahpF::kan* Δ(*katG::*Tn*10*)1 Δ(*katE12::*Tn*10*)	Laboratory Stock
BL21 (DE3)	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	Novagen
Plasmids	Relevant characteristics	Reference
pET16b	overexpression vector	Novagen
pRpe-His10	pET16b::*rpe*	This study

Table 2.2: Strain list. These strains and plasmids were used in the Rpe study.

2.6 FIGURES

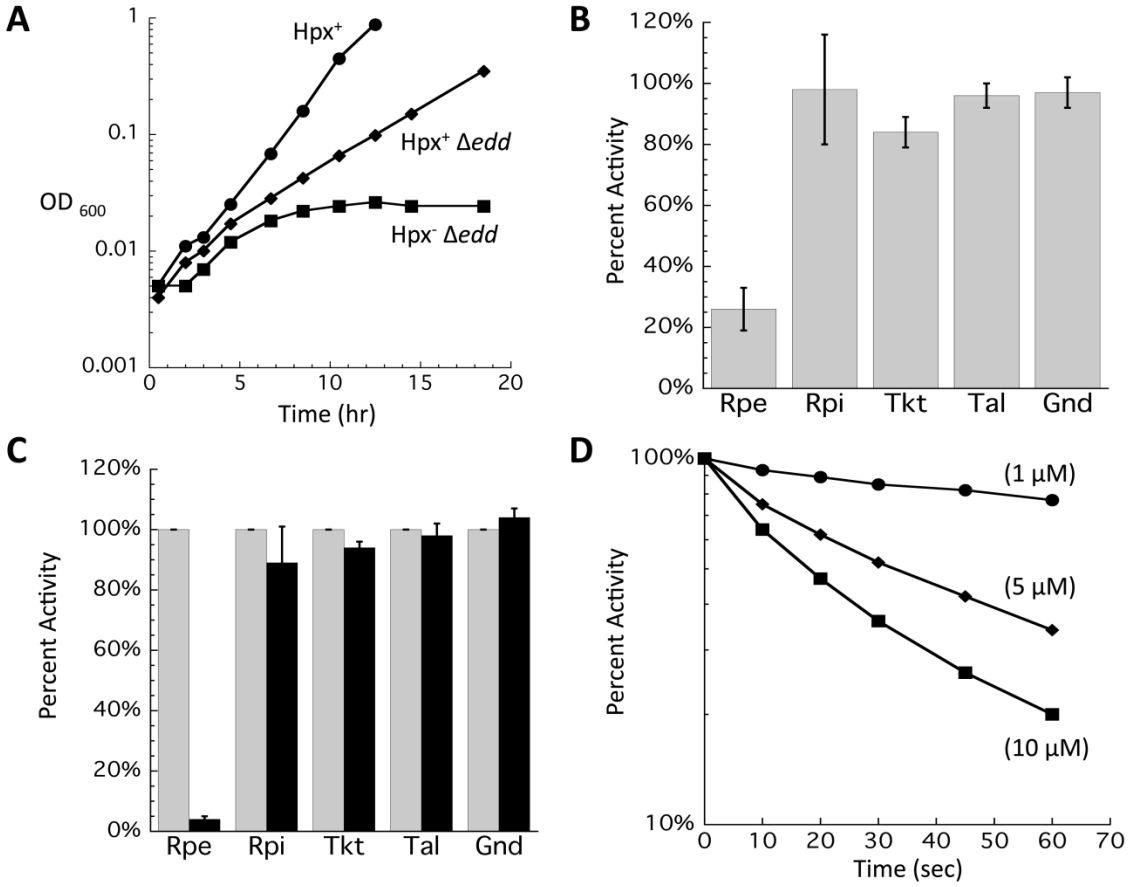

Figure 2.1: Hpx⁻ Δ*edd* cells cannot grow on gluconate because of damage to Rpe. (A) After anaerobic preculture, cells were diluted into aerobic minimal gluconate medium at time 0. Data presented are representative of multiple experiments. **(B)** Activities measured in extracts prepared from Hpx⁻ Δ*edd* cells grown aerobically. Values were normalized to those of anaerobic cells. Gnd, 6-phosphogluconate dehydrogenase; Rpi, ribose-5-phosphate isomerase; Tal, transaldolase; Tkt, transketolase. **(C)** Activities measured before (gray bars) and after (black bars) 10 μM H_2O_2 was added to cell extracts prepared from anaerobically grown Hpx⁻ cells. Error bars represent SD from the mean of three independent experiments. **(D)** Time course of the inactivation at 0 °C of Rpe in cell extracts treated with the indicated concentrations of H_2O_2. Extracts were prepared from anaerobically grown Hpx⁻ cells.

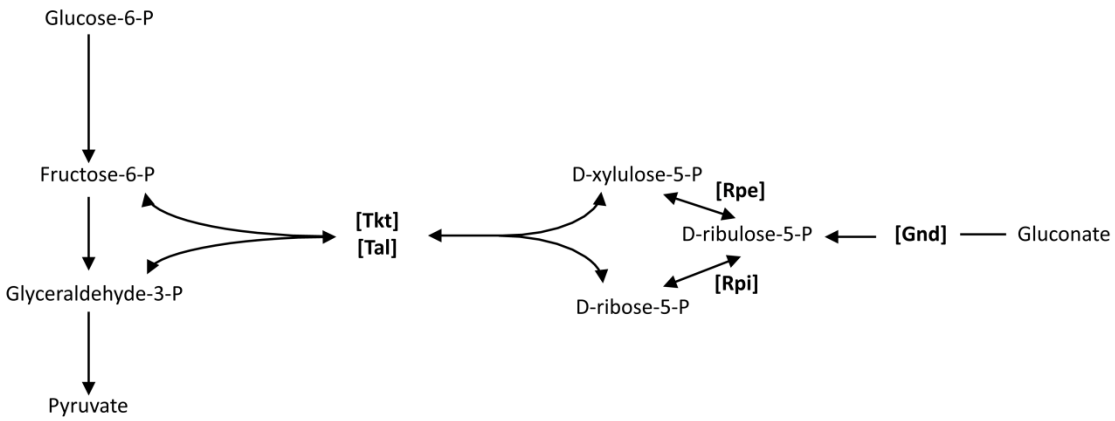

Figure 2.2: The pentose–phosphate pathway. Relevant intermediates are listed, with key enzymes denoted in brackets. Arrows indicate direction of each reaction.

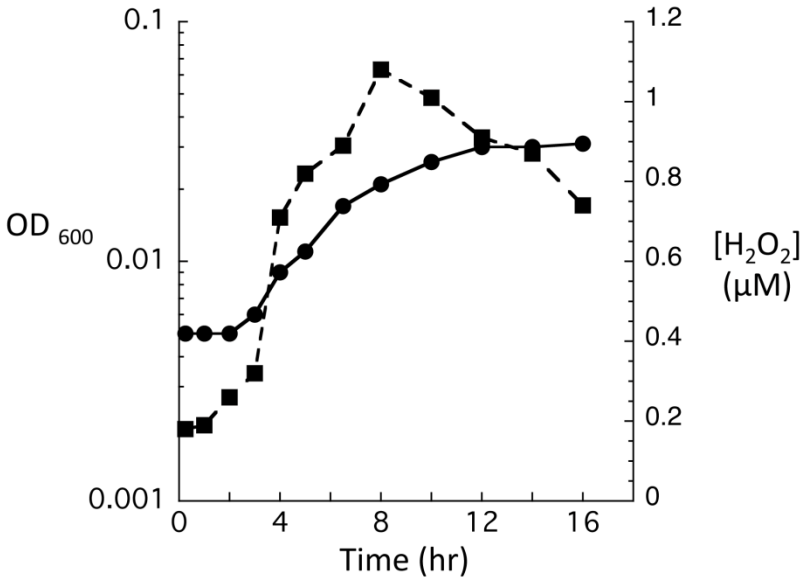

Figure 2.3: H_2O_2 accumulation during aerobic growth of the Hpx⁻ strain. After anaerobic preculture, cells were diluted into aerobic minimal gluconate medium at time 0. Growth was monitored (circles), and aliquots were removed for determination of H_2O_2 concentration (squares). H_2O_2 equilibrates across the cell membranes so that extracellular and intracellular concentrations are essentially equivalent. Data presented are representative of multiple experiments.

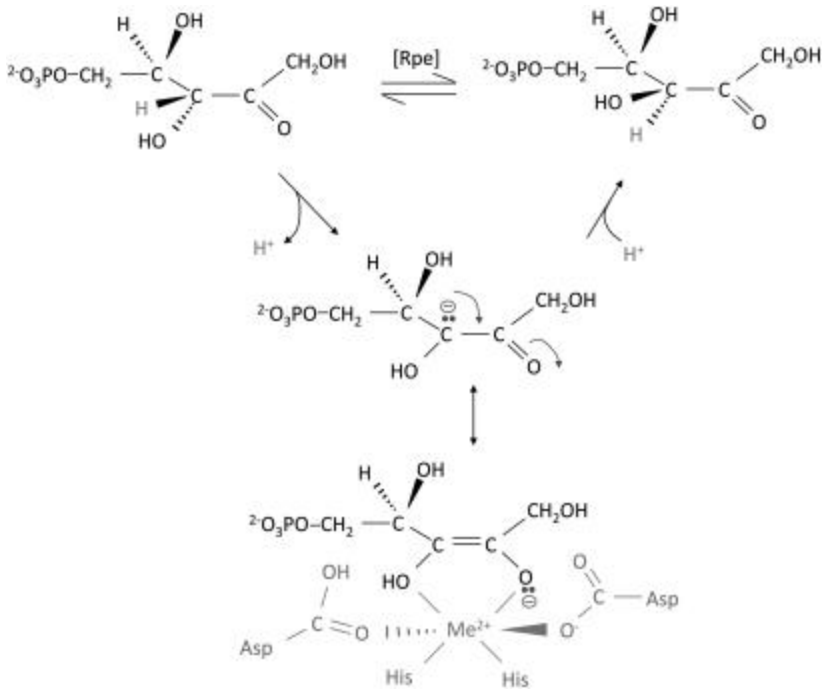

Figure 2.4: Rpe active site structure and mechanism. Rpe interconverts ribulose-5-phosphate and xylulose-5-phosphate by abstracting a proton from one face of the C-3 carbon and then adding a proton to the opposite face. The metal coordinates substrate and stabilizes the intermediate oxyanion [25].

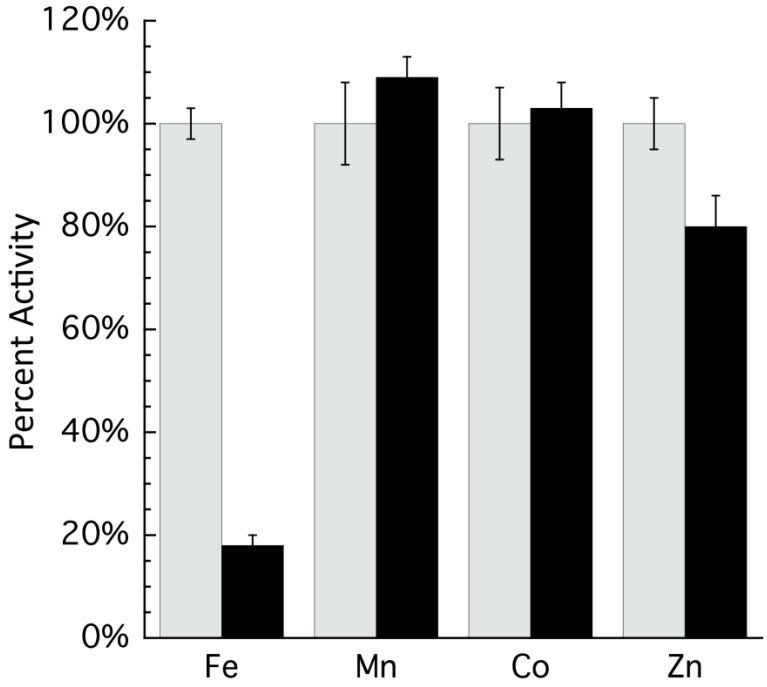

Figure 2.5: Sensitivity of Rpe metalloforms to H_2O_2 in vitro. Pure Rpe was metallated with the indicated metals. Activity then was measured before (gray bars) and after (black bars) treatment with 500 μM H_2O_2 for 2 min. Error bars represent SD from the mean of three independent measurements.

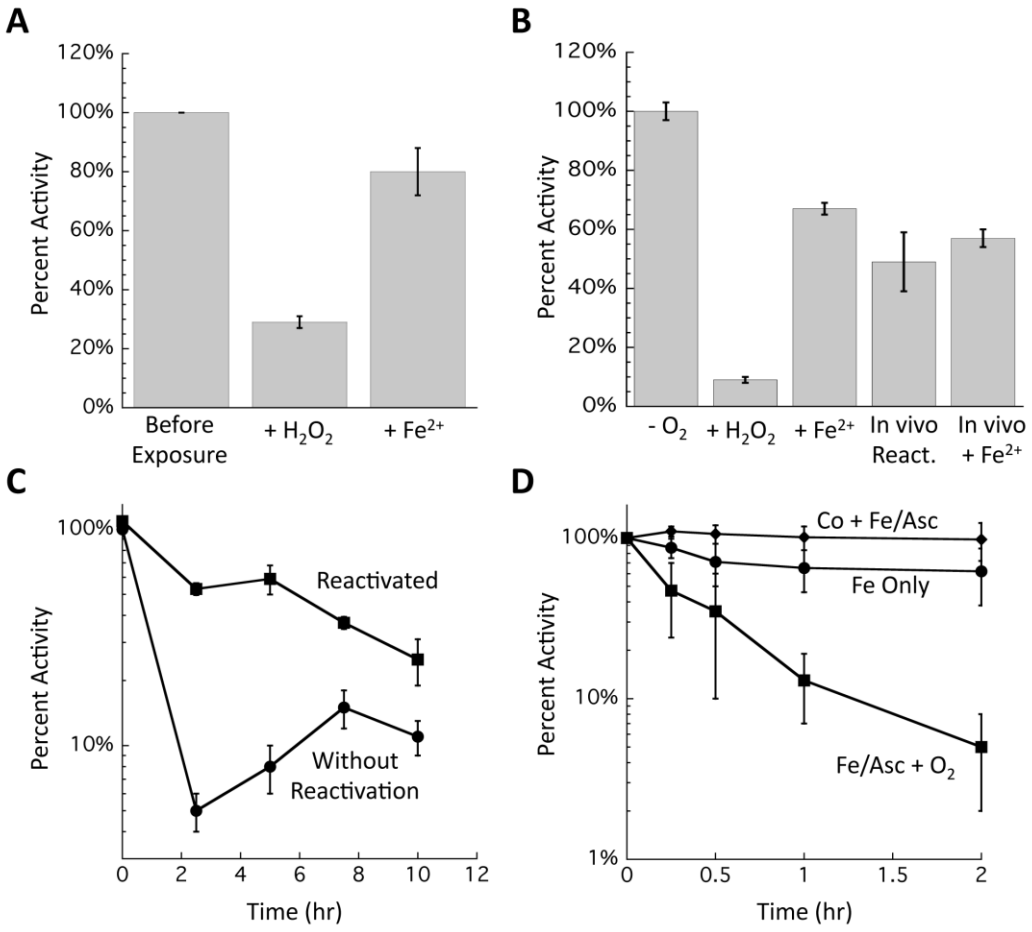

Figure 2.6: Reversibility of Rpe inactivation. (A) A single cycle of in vitro damage is mostly reversible. Pure Rpe was metallated with 100 μM Fe^{2+} before being treated with 200 μM H_2O_2 for 5 min. Catalase and DTPA were added to remove Fe and H_2O_2, and Rpe was assayed. Damaged enzyme then was reactivated by anaerobic incubation with 100 μM Fe^{2+} and 500 μM ascorbate. **(B)** Brief damage in vivo is mostly reversible. Hpx^- cultures were grown in anaerobic medium, and chloramphenicol then was added to block further protein synthesis. Cells then were aerated and exposed to H_2O_2 (100 μM) for 10 min. Cultures were returned to the anaerobic chamber, treated with catalase, and assayed before (+H_2O_2) or after (+Fe^{2+}) in vitro reactivation. In vivo reactivation was measured by incubating cells for an additional 20 min after the termination of H_2O_2 stress. **(C)** Protracted stress irreversibly damages Rpe in vivo. Anaerobic cells were diluted into aerobic minimal gluconate medium at time 0. At indicated time points Rpe activity was measured before and after in vitro reactivation with Co^{2+}. **(D)** Repetitive Fenton chemistry irreversibly damages Rpe in vitro. Pure Rpe was metallated with Fe^{2+} or Co^{2+}. After 10 min, samples were diluted into aerobic buffer containing Fe^{2+} and ascorbate to trigger cycles of H_2O_2 damage and remetallation by Fe^{2+}. At indicated time points, aliquots were removed, damage was terminated by the addition of catalase and DTPA, and Co^{2+} was added to activate the remaining functional apoenzyme. Error bars represent the SD from the mean of three experiments. The enzyme is not inactivated if the active site is occupied by Co.

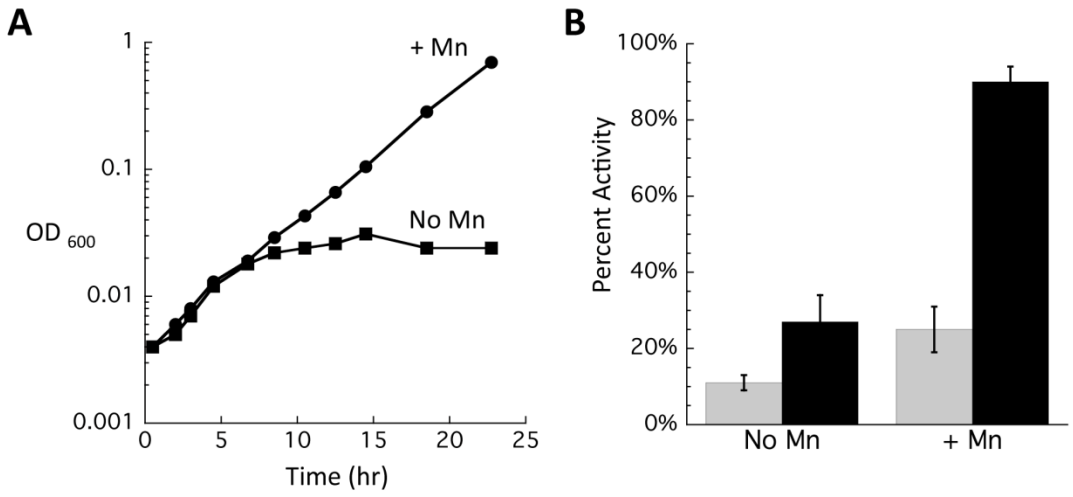

Figure 2.7: Mn supplements protect Rpe against irreversible damage in vivo. (A) After anaerobic preculture, cells were diluted at time 0 into aerobic minimal gluconate medium with or without 50 μM $MnCl_2$. Data are representative of multiple growth experiments. **(B)** Cells were handled as in *A* except that aerobic inoculum was to 0.05 OD. At various times, aliquots were taken, and Rpe activity was measured before and after reactivation with Co. Time 0 is an anaerobic time point. Error bars represent the SD from the mean of three independent experiments.

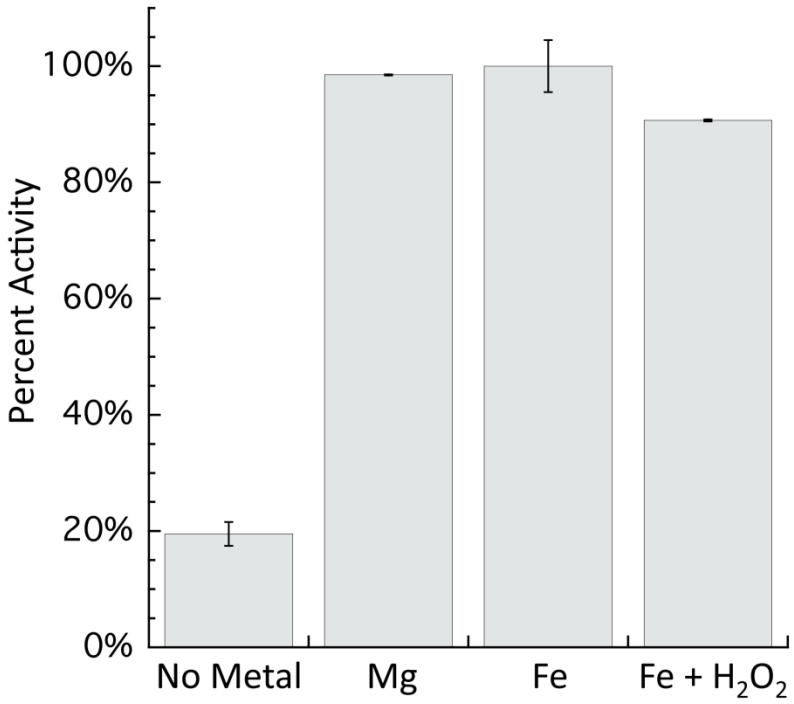

Figure 2.8: Transketolase and necessity of solvent exposure for metal sensitivity to chelation and to H_2O_2. Metals were added to pure transketolase (Sigma) before the addition of TPP and 500 μM H_2O_2. After mixing, samples were incubated for 5 min and then assayed.

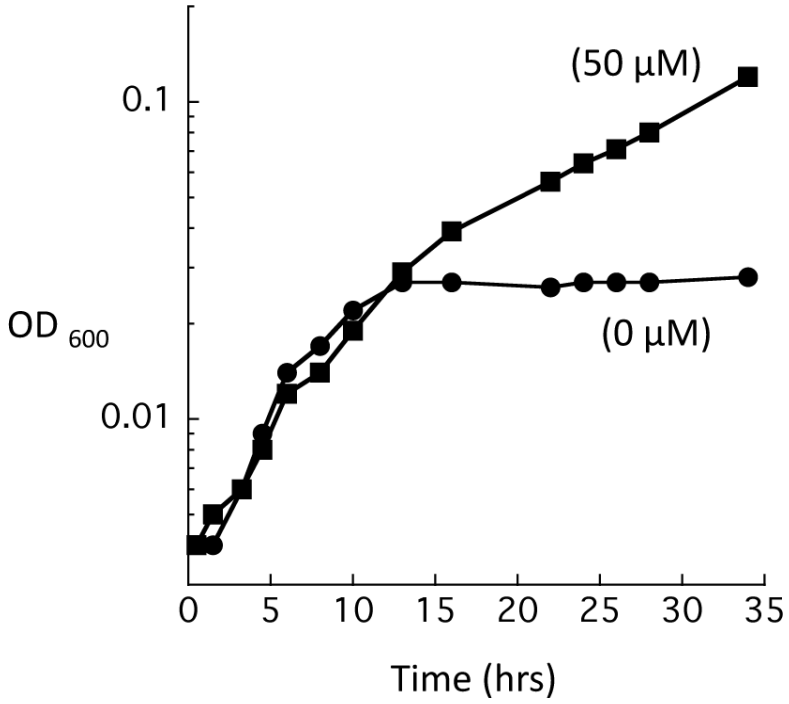

Figure 2.9: Co supplements allow Hpx$^-$ *edd* cells to grow on gluconate. After anaerobic preculture, cells were diluted into aerobic minimal gluconate medium with or without various concentrations of $CoCl_2$ at time zero. Concentrations of $CoCl_2$ used were: 0 μM (circles) and 50 μM (squares).

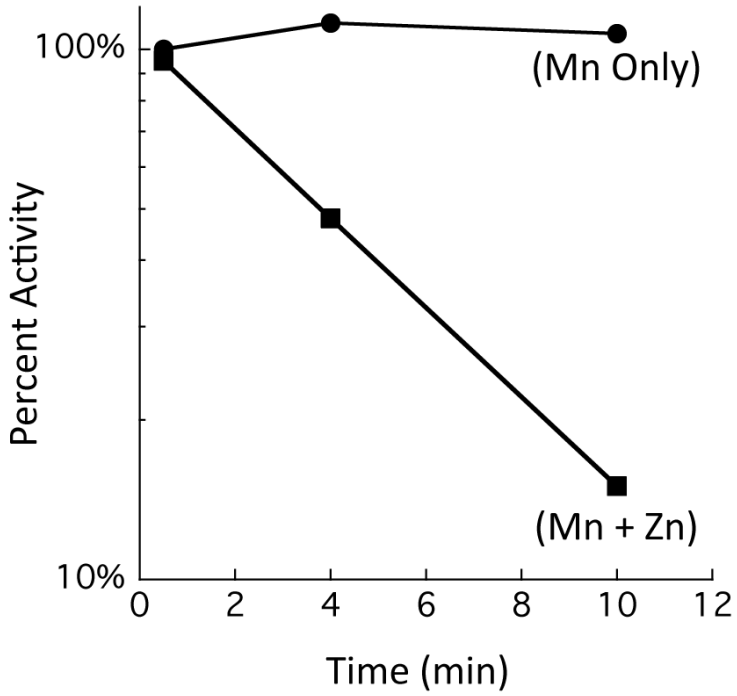

Figure 2.10: Zn^{2+} outcompetes Mn^{2+} for the Rpe active site. The activity of pure Rpe premetallated with Mn was monitored with (squares) or without (circles) the subsequent addition of Zn. Two hundred μM Mn^{2+} and 200 μM Zn^{2+} were present during the incubation period. Mn equilibrates between the active site and the bulk solution; when present, Zn deactivates the enzyme by binding irreversibly to the active site of the transient apoenzyme.

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CHAPTER 3: H_2O_2 IMPOSES AN AROMATIC AMINO ACID AUXOTROPHY ON *ESCHERICHIA COLI* BY DAMAGING DAHP SYNTHASE

3.1 INTRODUCTION

In 1900, when Loew first discovered catalase, an enzyme that could scavenge H_2O_2, the field of oxidative stress was born [43]. Although researchers recognized that there must be a reason why cells would want to rid themselves of this oxidant, years went by before real insights into its toxicity were realized. It would take more than 70 years for another reactive oxygen species scavenger, superoxide dismutase (SOD), to be discovered [46]. When McCord took an inventory of what organisms possessed these two scavenging systems, he found that in his sample set only aerobes possessed catalases and SODs [47]. This led some workers to conclude that the reason for the existence of obligate anaerobes was their lack of enzymes capable of detoxification of reactive oxygen species. At the time, this conclusion seemed valid, and this idea subsequently found its way into a number of textbooks. However, with the coming of the genomic age and further studies on enzymes capable of scavenging H_2O_2 and $O_2{\cdot}^-$, it has become apparent that anaerobes in fact do possess a number of scavenging enzymes, including peroxidases and superoxide reductases [37, 44, 51]. Thus, it remains a mystery as to why anaerobes cannot tolerate oxygenated environments, although it may be that oxygen itself poses the real threat.

The ubiquitous existence of scavenging systems for reactive oxygen species (ROS) implies a universal requirement to eliminate these toxic compounds. Indeed, there are a number of sources from which an organism may be exposed to these compounds. For example, the immune responses of plants and animals, including the oxidative burst of macrophages and neutrophils, bombard invading microorganisms with a bolus of reactive oxygen species, including H_2O_2 and $O_2{\cdot}^-$ [8, 21, 48, 55]. Some bacteria (such as lactic acid bacteria) are even capable of producing these oxidants to poison their

competitors [23, 60, 62]. Other organisms, including plants and some bacteria, secrete redox-cycling drugs like juglone and phenazines that, when taken up by organisms in the same environment, produce $O_2\cdot^-$ and H_2O_2 which are toxic to the competitors [34, 66]. Competing organisms are not the only sources of reactive oxygen species. The environment itself can be a source of H_2O_2, generated from the oxidation of organic molecules. Perhaps the most pertinent source of reactive oxygen species is the organism itself. $O_2\cdot^-$ can be generated within the cell as an inadvertent byproduct of aerobic metabolism when molecular oxygen picks up an electron from flavins [33, 49]. Subsequent dismutation forms H_2O_2 [10, 49]. In *E. coli*, H_2O_2 generated by this mechanism can accumulate to *ca*. 1 μM when H_2O_2 scavenging systems have been deleted [57].

Because there are so many avenues by which cells can come into contact with reactive oxygen species, it is necessary for organisms to have a way to respond to these toxins. In the case of H_2O_2, this response is mediated by OxyR. OxyR is a transcriptional regulator with an active-site cysteine residue that is poised to react with H_2O_2 [12, 41]. When this occurs, OxyR undergoes a conformational change that allows it to activate a number of genes, including ones encoding scavenging systems, Fe-S cluster assembly and repair systems, and manganese import, to name a few [69]. There is a similar response to redox-cycling drugs which produce $O_2\cdot^-$, via the SoxRS system [22, 42]. SoxRS was once thought to respond to $O_2\cdot^-$ directly, but recent work has shown that this response may not be so specific to $O_2\cdot^-$, but more likely a response to the redox cycling drugs [26, 39]. SoxRS is a two- component system where the sensor, SoxR, contains an oxidizable Fe-S cluster. When the cluster is oxidized, SoxRS activates transcription of members if the regulon [53]. These member proteins include Mn-SOD and a number of drug efflux and detoxification systems. These responses help cells to tolerate these unavoidable reactive oxygen species.

In order to better understand why scavenging systems play such a crucial role in the ability of organisms to survive reactive oxygen species, it is important to understand what the targets of these oxidants are. To do this, studies have taken advantage of mutants that either lack cytosolic SODs (SOD mutants) or lack both catalases and

peroxidase (Hpx^-) and are unable to scavenge these reactive oxygen species. Hpx^- cells, as mentioned previously, accumulate up to 1 μM H_2O_2, which is enough to poison themselves [58]. Several phenotypes result from H_2O_2 exposure. First is DNA damage, which is probably the most consequential of all the phenotypes. This occurs when the Fenton reaction takes place on the face of DNA, resulting in hydroxyl radical formation and oxidation of the DNA itself. If unchecked, the accumulation of DNA lesions can lead to loss in cell viability [52]. A second class of H_2O_2 target includes solvent-exposed Fe-S cluster dehydratases. These proteins have a [4Fe-4S] cluster where the fourth Fe atom is not fully coordinated but is free to bind and activate substrate. Damage to these proteins, though they require addition of H_2O_2 beyond 1 μM to become apparent, causes growth phenotypes such as a leucine auxotrophy, failure of the Isc Fe-S cluster assembly system, and an inability to grow on TCA cycle intermediates [35, 36]. In each case, the Fenton reaction occurs at the site of the under-coordinated Fe atom in the Fe-S clusters of the dehydratases. Finally, the newest targets of H_2O_2 to be discovered are mononuclear Fe proteins. Examples of these proteins include ribulose-5-phosphate 3-epimerase (Rpe), peptide deformylase (Pdf), cytosine deaminase (Cda), and threonine dehydrogenase (Tdh) [3]. Each of these proteins uses a solvent-exposed Fe atom to bind and activate substrate in a way analogous to the Fe-S cluster in dehydratases. This solvent exposure also makes them vulnerable to oxidation by H_2O_2. Again, the damage is due to Fenton chemistry at the active-site Fe atom.

SOD mutants have similar phenotypes due to damage to a number of the same proteins. While $O_2^{\cdot -}$ does not directly damage DNA, SOD mutants do experience a higher incidence of DNA damage than wild-type cells. This higher incidence of DNA damage is likely facilitated by increased levels of Fe, which has been released from damaged Fe-S proteins, available to participate in the Fenton reaction on the face of the DNA [30]. SOD mutants are also branched-chain amino acid auxotrophs, which is due to damage to the Fe-S clusters of the same dehydratases responsible for failure of the pathway in Hpx^- cells [18, 40]. Mononuclear Fe enzymes are not immune to damage by superoxide either. However, while Fenton chemistry in Hpx^- cells leads to irreversible enzyme damage, the failure of these proteins to function properly in SOD mutants

appears to be caused by mismetallation with Zn. This was observed with Rpe and Tdh [27].

While scavenging reactive oxygen species will remove the stress, it seems that the stress can be tolerated by import of Mn [1, 11, 31, 38, 59, 65]. In fact, almost all phenotypes, in both Hpx^- and SOD mutants, can be relieved by addition of Mn to the growth medium (Anjem & Imlay, unpublished data). While damage to DNA cannot be prevented by Mn, damage to Rpe, Pdf, Cda, and Tdh can be avoided if these proteins are metallated with Mn [3]. When Mn is in the active site, most of these proteins retain a large percentage of their Fe-loaded activity. While Fe is readily oxidized by either $O_2{\cdot}^-$ or H_2O_2, Mn is not. This allows the enzyme and by extension, the pathway, to function during oxidative stress, albeit at a slightly lesser rate. Mn has also been observed to prevent H_2O_2, and to a lesser extent $O_2{\cdot}^-$ from causing high levels of Fe-S cluster damage, although the mechanism by which Fe-S clusters are protected by Mn remains unclear (Anjem & Imlay, unpublished data).

One additional phenotype that has been described in both SOD and Hpx^- mutants is an auxotrophy for aromatic amino acids [9, 35]. While this phenotype has been known for years, there have been no compelling explanations for the cause of this auxotrophy. Although one study has suggested that the phenotype in SOD mutants is due to damage to the reaction intermediate of transketolase, these results may not be the real explanation [9]. Until a few years ago, there were no obvious candidates other than transketolase. However, recent studies identifying mononuclear Fe proteins as novel targets of these reactive oxygen species shed new light on this problem. In this study, I report that DAHP synthase, the first enzyme in the aromatic amino acid biosynthetic pathway (shikimic acid pathway), is a mononuclear Fe enzyme that is damaged by H_2O_2 and $O_2{\cdot}^-$ and is the target responsible for the aromatic amino acid auxotrophy. Furthermore, Mn can protect DAHP synthase from both oxidants by replacing Fe in the active site of the protein.

3.2 RESULTS

3.2.1 DAHP synthase is damaged by H_2O_2 in vivo

More than a decade ago, it was determined that *E. coli* strains that cannot scavenge H_2O_2 gradually accumulate about 1 μM H_2O_2 [58]. This Hpx^- strain has been shown to have a number of growth deficiencies, but the cause of the most sensitive phenotype, an auxotrophy for the aromatic amino acids (Figure 3.1), has remained elusive. In fact, less than 0.3 μM H_2O_2 is necessary to elicit this growth defect (Figure 3.2), which requires that all three aromatic amino acids (phenylalanine, tryptophan, and tyrosine) be added to the medium in order to fully restore growth to wild-type levels. This requirement implied failure of the common pathway for aromatic biosynthesis, rather than damage to the branch pathways for the individual amino acids.

The common aromatic biosynthetic pathway, the shikimic acid pathway, begins with phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) as substrates and progresses through a series of seven reactions, leading to the production of chorismate (Figure 3.3). Chorismate is the end product that branches not only to the individual biosynthetic pathways for the three aromatic amino acids but also to those for folate, ubiquinone, menaquinone, and siderophores. In order to narrow down where the point of damage to the pathway was occurring, I added the central intermediate, shikimate, to growth medium to determine whether the block was upstream or downstream of shikimate. When shikimate was added, Hpx^- cells were able to grow as quickly as when all three aromatic amino acids were provided (Figure 3.4). This indicated that the block in the pathway was upstream of shikimate. Recently I identified a new class of proteins that are targets of H_2O_2 – mononuclear iron enzymes. These proteins use a single solvent-exposed iron atom to bind and activate substrate. The first enzyme of the common biosynthetic pathway, 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase, is one such protein. This activity is supplied by 3 isozymes, and each is believed to be cofactored by iron in in vivo based on atomic absorption spectra of purified DAHP synthases [45].

When I exposed extracts to H_2O_2 in vitro, I found that the protein was acutely sensitive to H_2O_2. The DAHP synthase activity fell below 10% of that of the untreated activity (Figure 3.5). Control experiments were performed to determine which of the three isozymes of DAHP synthase were contributing to the activity, as well as the sensitivity of each to H_2O_2. This was done by feedback inhibition of two of the three isozymes of DAHP synthase by addition of combinations of two aromatic amino acids. In vitro measurements indicated that all three isozymes were sensitive to H_2O_2 treatment (data not shown) and that the phenylalanine-responsive isozyme, AroG, accounted for most of the activity, as has been described previously [15, 64]. This demonstrated the ability of H_2O_2 to directly damage DAHP synthase, but does this actually occur in the cell during H_2O_2 stress? To investigate this I measured the activity of DAHP synthase from aerobic cultures of Hpx^- cells over time. While wild-type cells maintained a constant level of activity, Hpx^- cells experienced a progressive decrease in DAHP synthase activity, to a final level of only 20 to 25% of the wild-type activity (Figure 3.6). This result confirmed that DAHP synthase is sensitive to H_2O_2 in vivo.

3.2.2 DAHP synthase is a mononuclear iron enzyme that is sensitive to Fenton chemistry

Other mononuclear iron proteins have been shown to be activated by a number of different metals. To determine what metals are able to activate DAHP synthase, as well as which metals might sensitize it to damage by H_2O_2, DAHP synthase was purified using a His tag, which was cleaved off after purification. Because the AroG isozyme of DAHP synthase comprises the largest percentage of total activity under normal growth conditions [15] [64], I chose to use this isozyme in our in vitro studies. Published assays have shown that cobalt is able to activate DAHP synthase [63]. However, *E. coli* does not import cobalt; therefore it is unlikely that cobalt is physiologically relevant with respect to in vivo metallation of DAHP synthase. Three physiologically relevant metals, iron, manganese, and zinc, were added to apo-DAHP synthase and assayed for their ability to activate the protein. Each of the three metals tested was able to activate DAHP synthase, to varying degrees. Iron yielded protein with the highest activity, whereas

manganese was able to activate DAHP synthase to about 70% of the iron-activated sample (Figure 3.7). Zinc appeared to be a less suitable metal for activation. While DAHP synthase did become activated after zinc addition, the maximum level of activity was only 20% of the levels obtained using iron (Figure 3.7). These three metalloforms were also assayed for their sensitivity to H_2O_2. When pure protein was challenged with 100 μM H_2O_2, only the iron-metallated isoform was sensitive to H_2O_2, losing almost 90% of its activity (Figure 3.7). This result suggests that iron metallates DAHP synthase in vivo.

While the use of pure DAHP synthase to determine the kinetics of inactivation as well as the mechanism of damage would have been the most desirable method, the pure protein was not stable enough to facilitate such experiments. Therefore, cell extracts from anaerobically-grown Hpx^- cells were used to determine the rate of inactivation of DAHP synthase by H_2O_2. The apparent inactivation rate constant was found to be about 500 – 1000 M^{-1} s^{-1} at 0°C, which is roughly 5-fold lower than the inactivation rate constants (4000 – 5000 M^{-1} s^{-1} at 0°C) determined for other mononuclear iron proteins, such as Rpe.

3.2.3 Damage to DAHP synthase in vivo is different than damage in vitro

In order to determine the mechanism by which H_2O_2 damages DAHP synthase, enzyme that was damaged in vivo was reactivated in vitro by addition of iron and the reducing agent tris(2-carboxyethyl)phosphine (TCEP). TCEP was included because one of the ligands responsible for coordination of the metal ion in the active site is a cysteine residue (Figure 3.8). It seemed plausible that if the Fenton reaction were occurring while iron was bound to the cysteine, oxidation of the sulfhydryl might occur, requiring reduction of the resultant sulfenic acid before reactivation could occur. When DAHP synthase activity was diminished through the aeration of Hpx^- cells, the activity could be recovered completely by the addition of iron in vitro without the need for reductant (Figure 3.8). It has been reported that mononuclear Fe proteins accumulate Zn in their active sites during $O_2^{\cdot -}$ stress [27]. In order to confirm determine whether this was also occurring during H_2O_2 stress, penicillamine, a metal chelator with preference for zinc,

was also added to remove any metals that may have remained in the active site. When this treatment was followed by activation with iron or zinc, lower activity was recovered (Figure 3.8). While it is unclear why full reactivation was not achieved after this treatment, it is clear that DAHP synthase is not poisoned due to mismetallation by zinc during the H_2O_2 stress.

In contrast, when DAHP synthase was inactivated in vitro using 100 µM H_2O_2 in the presence of chelator to prevent remetallation, only 13% of the activity could be recovered with iron addition (Figure 3.9). When TCEP was included, up to 24% of the activity was recovered, implying that the cysteine residue was partially oxidized in vitro during the H_2O_2 challenge. In order to determine whether the cysteine residue itself was sensitive to oxidation, apo-DAHP synthase was prepared by treating Hpx^- cell extracts with chelator for 30 minutes. After the apo-protein had been generated, the extracts were treated with different amounts of H_2O_2 to determine the inactivation rate constant for the cysteine residue. The apparent rate constant measured for the cysteine residue itself was only *ca*. 5 M^{-1} s^{-1}, which approximates the values obtained for other thiols [13, 16, 25, 61, 68]. Thus, it appears that there may be some protective component in the cell, such as reducing systems or manganese, which are either too dilute or otherwise inactivated in the extract, thereby preventing protection of the thiol.

3.2.4 Manganese is able to protect DAHP synthase by replacing iron in the active site

The ability of manganese to protect cells from oxidants has been well established, including with respect to protection of mononuclear iron enzymes from H_2O_2. For example, Rpe activity was protected by addition of manganese, as was described earlier, and the activities of other mononuclear iron proteins have been protected by manganese addition as well [3]. Therefore, I tested whether manganese supplements would allow Hpx^- mutants to grow aerobically without aromatic amino acids. As seen in Figure 3.10, addition of 50 µM manganese was able to completely suppress the growth phenotype. In fact, as little as 0.5 µM manganese was able to restore growth to the Hpx^- strain (Figure 3.11). Evidence that this suppression is the result of protection of DAHP synthase by

metallation by manganese was obtained by assaying DAHP synthase activity taken from manganese-fed Hpx$^-$ cells. The DAHP synthase activity in these cells was between 60 and 70% that of wild-type cells (Figure 3.6), which matched almost perfectly with the amount of activity obtained when pure DAHP synthase was metallated with manganese versus iron (Figure 3.7). In addition, when the manganese importer, MntH, was deleted from the Hpx$^-$ strain, DAHP synthase activity was even lower than in the Hpx$^-$ strain itself, falling to less than 5% of the wild-type activity, near the limit of detection (Figure 3.6). Thus, it appears that manganese can protect DAHP synthase from damage by H_2O_2.

Other evidence the supports the idea that manganese protection of DAHP synthase is by protecting the active site of the protein is provided by additional media supplementation experiments. Manganese itself was not the only metal supplement that was able to protect DAHP synthase. When cobalt was added to growth medium, there was some relief of the aromatic amino acid auxotrophy as seen by an improvement in growth (Figure 3.12). While cobalt is toxic itself at higher concentrations, there was improvement in growth rate at concentrations up to 50 μM cobalt. Cobalt can activate DAHP synthase, but it does not scavenge H_2O_2, thus protection by cobalt must be by replacement of iron in the active site of DAHP synthase. Furthermore, addition of an iron chelator, deferoxamine, also restores growth to aerobically-grown Hpx$^-$ cells (Figure 3.13). Because iron chelation starves the cell for iron, enzymes must be metallated by alternative metals, such as manganese. These other metals will not be sensitive to oxidation by H_2O_2 and will thereby protect DAHP synthase from damage. Taken together, these data support the idea that the mechanism of manganese protection of DAHP synthase from H_2O_2 is by replacing iron in the enzyme active site.

3.2.5 *E. coli* attempts to compensate for diminished DAHP synthase activity by increasing expression

It seemed likely that *E. coli* would make an attempt to compensate for decreased output of the aromatic amino acid biosynthetic pathway by inducing transcription of genes in the pathway. DAHP synthases are the first proteins in the biosynthetic pathway, and expression of the proteins is known to be controlled by TrpR or TyrR, transcriptional

regulators in response to levels of tryptophan and tyrosine, respectively. I hypothesized that expression of one or more isozymes of DAHP synthase would be induced during H_2O_2 stress. To determine whether the levels of DAHP synthase expression were being affected under these conditions, I constructed single-copy transcriptional fusions of the *aroG* or *aroF* promoters to the *lacZ* gene and inserted these into the λ attachment site of the *E. coli* chromosome. While the transcription of *aroG* was not affected by H_2O_2 stress, likely due to a lesser degree of regulation by TyrR, transcription of the *aroF* gene, which encodes the tyrosine-responsive isozyme of DAHP synthase, increased by 7.5-fold over wild-type levels (Figure 3.14). The *aroF* gene is regulated by TyrR, a tyrosine-sensitive repressor, so the levels of transcription were measured in a Δ*tyrR* mutant as well. In the *tyrR* null strain, *aroF* expression increased about 28-fold (Figure 3.14). Thus, induction in response to H_2O_2 is not complete, but it is apparent that expression increases as a way to compensate for diminished activity of the pathway. I also investigated whether the addition of manganese would return expression of *aroF* to wild-type levels, since the growth phenotype is suppressed by manganese supplements. While the expression did diminish some (from 7.5 to 5-fold above wild-type), there must still be enough injury to warrant an increase in expression to maintain adequate flux through the aromatic biosynthetic pathway (Figure 3.14).

3.3 DISCUSSION

Early life forms arose and developed in an anoxic, Fe-rich environment [2, 5, 32]. These early predecessors of modern-day organisms built their metabolism around what was readily available to them. This led enzymes catalyzing reactions that could be more easily achieved by incorporating Fe, either as a redox moiety, or as a way to facilitate substrate binding and activation, to be built with Fe as an integral cofactor. While Fe is very good at surface chemistry and would have achieved these goals for early life in the absence of oxygen, photosystem II brought with it a need for organisms to adapt. The Fe that made the lives of early organisms possible was now a source of vulnerability. In

some enzymes the necessity of solvent exposure of Fe for interaction with substrate also meant exposure to oxidants.

As more enzymes are studied, I find more frequently that Fe remains an integral part of aerobic life. Despite the vulnerability to reactive oxygen species posed by this metal, life finds a way to make it work. DAHP synthase represents the fifth mononuclear protein I have studied that uses Fe under normal physiological conditions despite being sensitive to oxidation by both H_2O_2 and $O_2\cdot^-$ (Figures 3.5 & 3.6) [3, 27]. It is not difficult to comprehend why these enzymes would remain despite their inactivation by reactive oxygen species. The metabolic processes employed by early organisms evolved over billions of years, and while the rise of atmospheric oxygen levels occurred gradually over time, it was much easier for organisms to develop new strategies to counteract the effects of these new reactive oxygen species than to completely rewrite the book on central metabolism.

3.3.1 H_2O_2 and $O_2\cdot^-$ damage mononuclear iron proteins

To combat the onslaught of reactive oxygen species, organisms evolved scavenging systems to help cope with their effects. As a result, normal physiological levels of reactive oxygen species are kept at bay by the activities of SODs, catalases, and peroxidases. However, under conditions of ROS stress, when organisms come into contact with high levels of $O_2\cdot^-$, H_2O_2, or redox-cycling compounds in the environment, these scavenging systems may not be enough. Increases in the steady-state levels of $O_2\cdot^-$ and H_2O_2 can still damage cells. Together with Rpe, Pdf, Cda, and Tdh, DAHP synthase represents another example of the vulnerability of mononuclear Fe proteins to reactive oxygen species. These mononuclear Fe proteins join Fe-S cluster proteins as the primary protein targets of both $O_2\cdot^-$ and H_2O_2. While $O_2\cdot^-$ and H_2O_2 appear to target the same classes of proteins, the mechanisms by which the damage occurs does not seem to be identical.

To date, all solvent-exposed Fe proteins studies have been damaged by H_2O_2 via the Fenton reaction. This leads to production of the ferryl radical intermediate, and

subsequently, the highly reactive hydroxyl radical. If multiple damage cycles occur, the hydroxyl radical can eventually irreversibly inactivate these proteins by oxidizing the polypeptide. In the case of DAHP synthase, it is possible that oxidation of the cysteine residue that coordinates the metal atom prevents repeated oxidation cycles from occurring. There is evidence that this may occur, as DAHP synthase damaged in vitro at lower concentrations of H_2O_2 can be partially reactivated by Fe with the use of a reductant. However, pure DAHP synthase loses the ability to be reactivated at high levels of H_2O_2 exposure, even with the use of reductants, implying that the cysteine residue can be oxidized beyond the sulfenic acid form. While this strategy leaves the protein in a temporarily inactive state at low levels of H_2O_2, when the stress is removed, the cell may be able to reduce the sulfenic acid back to the sulfhydryl and allow the protein to regain function.

On the other hand, $O_2{\cdot}^-$ does not merely inactivate DAHP synthase by oxidation of the Fe atom, but further complicates the problem by allowing mismetallation by zinc (Gu and Imlay, unpublished data). This appears to be the mechanism by which $O_2{\cdot}^-$ inactivates other mononuclear Fe proteins as well [27]. While this mechanism of damage is well supported and it is conceivable that another metal would be able to bind the metal site in these proteins, it remains unclear why this does not happen during H_2O_2 stress. In the case of DAHP synthase, oxidation of the cysteine residue may prevent other metals from making their way into the active site. However, not all mononuclear Fe proteins employ a cysteine residue to bind metal. Are these proteins also mismetallated? Further investigation is necessary to determine what the metallation status of other mononuclear Fe proteins is during H_2O_2 stress, but it is clear that this phenomenon does not occur in DAHP synthase.

3.3.2 The role of manganese in protection against oxidative stress

One mechanism by which cells attempt to protect themselves from reactive oxygen species is to induce scavenging systems to remove them. Another strategy is to limit Fe availability. Since Fe is responsible for DNA damage observed during both $O_2{\cdot}^-$ and H_2O_2 stress, this strategy makes sense. However, how are proteins that require Fe

going to function if this metal is sequestered? Most extant enzymes will have no problem under these circumstances, but enzymes that lose Fe will be affected. Relief of some SOD mutant phenotypes has been observed when Mn was added to the growth medium. While Mn cannot protect against DNA damage and has only a limited effect on SOD mutant phenotypes associated with Fe-S cluster damage, it clearly protects mononuclear Fe proteins from being mismetallated (Gu & Imlay, unpublished data). Despite this protective effect of Mn on SOD mutants, Mn import is not induced in response to $O_2^{\cdot -}$. However, MntH, the sole Mn transporter in *E. coli*, is a member of the OxyR regulon which is induced during H_2O_2 stress [69]. Under H_2O_2 stress conditions, Mn can protect against both Fe-S cluster phenotypes (Anjem and Imlay, unpublished data) as well as damage to mononuclear Fe proteins [3]. Some believed that Mn could scavenge reactive oxygen species itself, but this was shown not to be true in *E. coli* [4]. While it is unclear how Mn might protect Fe-S clusters, I suspect that it may be mediated by Fe sparing. However, the effect is direct in the case of mononuclear Fe proteins, as these enzymes can use Mn in place of Fe during stress conditions (Figure 3.6) [3].

So why do these proteins not use Mn all the time? Mn is able to activate many mononuclear Fe proteins, but provide less activity than does Fe. Additionally, these proteins seem to have a slightly higher affinity for Fe, probably because iron is generally better at binding ligands, according to the Irving-Williams series. The key in determining whether Mn or Fe will be used in these proteins seems to be based on their relative abundance and availability. Under normal circumstances, *E. coli* does not import Mn, so levels remain low, and proteins are metallated with Fe. However, under H_2O_2 stress conditions, Fe is oxidized and sequestered, while Mn is imported via MntH [24]. This causes levels of available Fe to fall, while the relative concentration of Mn increases, allowing Mn to serve as the activating metal in the absence of Fe. This may be why mismetallation by Zn is not a problem during H_2O_2 stress, but is during $O_2^{\cdot -}$ stress, since Mn is not imported under those conditions.

Some organisms have found ways around the problems posed by using Fe. One example, *B. burgdorferi*, which is the causative agent for Lyme Disease, does not require Fe at all [19, 54]. Lactic acid bacteria import extremely high amounts of Mn, allowing

them to produce H_2O_2 and withstand concentrations that rise to millimolar levels [6, 7]. Lactic acid bacteria can also employ isozymes of proteins that do not require Fe, or dispense with pathways requiring such enzymes altogether. The cost, in some cases, is that these organisms become auxotrophic for certain metabolites, but if their habitat provides these needs, those pathways become dispensable. One interesting question is whether eukaryotes have evolved in a similar fashion to low-Fe organisms with respect to metal specificity or use of alternative, metal-free isozymes. These questions – how do low-Fe organisms carry out reactions that are vulnerable to reactive oxygen species in *E. coli*, and have eukaryotes evolved to no longer require Fe for these processes – are foci of current work in this field. Answering these questions may help us to understand how these organisms can withstand oxidative stress so much more effectively than *E. coli*.

3.4 EXPERIMENTAL PROCEDURES

3.4.1 Reagents

Amino acids, antibiotics, catalase (from bovine liver), diethylenetriaminepentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), D-erythrose 4-phosphate sodium salt, phospho(enol)pyruvic acid tri(cyclohexylammonium) salt**,** ferrous ammonium sulfate hexahydrate, imidazole, manganese (II) chloride tetrahydrate, zinc chloride, trichloroacetic acid, sodium (meta) periodate, sodium arsenite, thiobarbituric acid, cyclohexanone, and 30% H_2O_2 were from Sigma. Tris-HCl was from Fisher.

3.4.2 Bacterial growth

Luria-Bertani (LB) medium contained 10 g tryptone, 10 g NaCl, and 5 g yeast extract per liter. Minimal medium was composed of minimal A salts [50] supplemented with 0.2% glucose, 0.5 µg/mL thiamin and 0.5 mM histidine. Other amino acids were added at 0.5 mM where indicated.

Anoxic growth was carried out in a Coy anaerobic chamber which contained an atmosphere consisting of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. Cultures were maintained at 37°C. Aerobic cultures were grown by shaking cultures vigorously at 37°C.

3.4.3 Strains and strain construction

A comprehensive strain list provided as table 3.5.1. Constructions in the Hpx^- background were carried out in an anaerobic chamber to ensure that no suppressor mutations were selected. Null mutations were constructed using the λ Red recombinase method [14]. All mutations were moved into the desired strain backgrounds by P1 transduction [50] and confirmed by PCR. Antibiotic cassettes were removed by transformation of pCP20 followed by removal of the plasmid [14].

Single-copy *lacZ* fusions to the *aroG* promoter were made by integration of the construct into the l attachment site. The native *aroG* gene was maintained on the chromosome [28, 29]. The promoter region was amplified using the primers: 5'-GCATCGCTGCAGTTCTTTCTCCTTTTTCAAAG-3' and 5'-GCATCGGAATTCCATGATGGCGATCCTGTTTA- 3' with PstI and EcoRI restriction sites, respectively. The modified CRIM plasmid, pSJ501, which contains a chloramphenicol resistance cassette, was used to allow anaerobic selections. The *aroG* promoter region was inserted into pSJ501 and confirmed by sequencing. The construct was then integrated into the chromosome and transduced into the desired recipient strains.

3.4.4 Growth curves

Cells were first grown overnight to stationary phase in anaerobic minimal glucose medium. Cultures were then inoculated to ~ 0.01 OD and grown anaerobically to 0.2 OD. Cells were then used to inoculate fresh aerobic minimal medium to 0.005 OD. Where indicated, the specified concentrations of $MnCl_2$, CoCl, or deferroxamine were added to aerobic growth medium.

3.4.5 DAHP synthase assays

3.4.5.1 In vivo H_2O_2 exposure

Cells for assays were grown in minimal medium without aromatic amino acids to ensure expression of DAHP synthase. Samples were collected by centrifugation of 50 mL culture for 10 min at 6,000 x g at 4°C. Supernatants were discarded and pellets were resuspended in 25 mL cold 0.1 M KP_i, pH 6.4. Samples were spun again for 10 min at 6,000 x g at 4°C. Supernatants were again discarded and pellets were moved into an anaerobic chamber and resuspend in 1 mL cold anaerobic 0.1 M KP_i, pH 6.4 + 9 mM PEP + 100 µM DTPA. Samples were then lysed by sonication for 1 minute using 3 second pulses. Extracts were then spun for 1 min at 20,000 x g at 4°C. Supernatants were then assayed immediately.

The assay used to determine DAHP synthase activity generates a cleavage product of DAHP by treatment with periodate which can be visualized by formation of a chromophore upon addition of thiobarbituric acid. The method described below is a variation of that described by Weissbach and Hurwitz [67] and modified by Doy and Brown [17].

Part I: Tubes were prepared anaerobically, containing 0.1 M KP_i, pH 6.4, 0.75 mM PEP and 0.6 mM E4P, unless otherwise indicated, and incubated at 37°C for 10 minutes (Total volume is 500 µL – extract volume). Treated extracts (100 µL extract + volume from additions, see below) were added to start the reaction. Reactions were incubated at 37°C for 1 min, then an aliquot of 150 µL was taken from the reaction and pipetted directly into a 1.5 mL tube containing 50 µL ice-cold 10% TCA. Samples were kept on ice while remaining samples were harvested until the final sample had been on ice for 5 minutes. Samples were then spun 10 min at 20,000 x g at 4°C to remove debris and removed from the anaerobic chamber.

Part II: 125 µL of each sample in TCA was pipetted into a glass test tube containing 125 µL 25 mM periodate in 0.075 M H_2SO_4, and the mixture was then incubated at 37°C for 30 min. Next, 250 µL 2% arsenite in 0.5 M HCl was added to each

sample. Samples were then shaken well for a few seconds by hand until the yellow color disappeared. Next, 1 mL 0.6% thiobarbituric acid in 0.5 M Na_2SO_4 was added to each sample and mixed well by shaking by hand for a few seconds. Samples were then closed by capping the tubes and heated vigorously (slowly boiling in a 110°C heating block) for 15 min, then placed in a water bath at room temperature for 5 minutes. 1 mL cyclohexanone was added to a 15 mL glass centrifuge tube for each sample before 1 mL of each reaction mixture was added to the centrifuge tubes. Samples were mixed well by shaking gently back and forth for a few seconds, then spun at 1000 x g at room temperature to separate layers. Absorbance of 500 µL of the top organic layer was measured at 549 nm using a Perkin-Elmer Lambda 25 spectrophotometer.

3.4.5.2 In vitro H_2O_2 exposure

Cultures were grown anaerobically overnight in minimal A glucose medium + thiamin, subcultured anaerobically for Hpx^- cells or aerobically for Kat^- cells [56] in the same medium, and grown to 0.2 OD. Cells were harvested as above, and extracts were prepared anaerobically in 9 mM PEP + 1 mM DTPA as above. Samples were challenged anaerobically +/- 100 µM H_2O_2 for 5 minutes before being added to the reaction mixture, as was done for in vivo exposure. The remainder of the assay was identical to in vivo exposure determinations of DAHP synthase activity.

3.4.5.3 In vitro reactivation of DAHP synthase

Reactivation of DAHP synthase damaged in vivo or in vitro was carried out identically to the description above, except that 100 µL extract was treated with 11 µL of 1 mM H_2O_2 for 5 minutes or 11 µL 50 mM $(NH_4)_2Fe(SO_4)_2$ +/- 1.1 µL 50 mM TCEP for 15 minutes. In addition, for protein damaged in vivo, 20 µL of 30 mM Penicillamine for 20 minutes. Penicillamine-treated samples were then treated with either 13.4 µL 50 mM $(NH_4)_2Fe(SO_4)_2$ or $ZnCl_2$.

3.4.5.4 Inactivation rate determination

Rates of inactivation of DAHP synthase were measured as for in vitro measurements, using Hpx^- cells, except that inactivation was carried out using 5, 20, or

100 µM H_2O_2 for 2 min before 10 µL of a 1:1000 dilution of catalase was added and activity was determined. These measurements were done in triplicate at 4, 10, and 20ºC.

3.4.6 Purification of DAHP synthase

DAHP synthase was purified by cloning of the *E. coli aroG* gene into the T7-containing pET42a vector using the forward primer 5'-CGACCTACCATGGGCAGCATCGAAGGTCGTATGAATTATCAGAACGACGATTTACGCATC-3', which included an NcoI restriction site, and the reverse primer 5'-GCTGGATGGATCCTTACCCGCGACGCGCTTTTACTGCATTC -3', which included a BamHI restriction site. After ligation, plasmids were transformed into DH5α and selected on LB plates containing 50 µg/mL ampicillin. One transformant found to have the correct *aroG* sequence was chosen for DAHP synthase purification. This plasmid was purified and transformed into *E. coli* strain BL21. This strain, containing plasmid pAroG-His was grown in LB glucose medium containing 50 µg/mL ampicillin to stationary phase, diluted into fresh LB glucose containing 50 µg/mL ampicillin to 0.006 OD, and grown at 37°C to 0.2 OD. This preculture was then used to inoculate 1 L fresh LB glucose containing 50 µg/mL ampicillin to 0.005 OD and cells were grown at 37°C to 0.6 OD. IPTG was then added to a final concentration of 0.4 mM, the culture was shifted to 15°C, and incubated for 18 hours.

Purification was performed as follows. Buffers used were: sample buffer (50 mM KP_i, pH 7.4, 0.5 M NaCl, 200 µM PEP, and 20 mM imidazole), elution buffer (50 mM KP_i, pH 7.4, 0.5 M NaCl, 200 µM PEP, and 0.5 M imidazole), cleavage buffer (50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 200 µM PEP, and 5 mM $CaCl_2$), and storage buffer (50 mM KP_i, pH 6.4, 200 µM PEP, and 2 mM EDTA). Cells were centrifuged and washed with 30 mL ice-cold sample buffer, resuspended in 20 mL ice-cold binding buffer, and sonicated. Cell extracts were cleared by centrifugation for 15 minutes at 4000 x g at 4°C. Two His Gravitrap pre-packed columns (GE Healthcare) were equilibrated aerobically at 4°C using 10 mL binding buffer. Each column was loaded with 5 mL cleared extract and washed with 10 mL sample buffer. Elution was conducted by adding 3 mL elution buffer to each column. This step was repeated to ensure all protein had been eluted from

the column. Eluates were pooled and 3 mL was dialyzed against 3 L cleavage buffer at 4°C for 8 hours.

To remove the 10-His tag, dialysate was cleaved using a Factor Xa Cleavage/Capture Kit (Novagen). The cleavage mixture contained 10 µL Factor Xa (2U/µL) and 990 µL dialysate per mL. This mixture was incubated at 4°C for 72 hours. Factor Xa was then removed according to kit instructions. Flowthrough from multiple columns were pooled together. Next, to remove uncleaved AroG-His10, a His Gravitrap column was equilibrated with 10 mL sample buffer at 4°C. Flowthrough from Factor Xa removal was then loaded onto the column and washed with 5 mL sample buffer. The resulting purified DAHP synthase was dialyzed against a 1000-fold excess of storage buffer for 8 hours at 4°C and stored on ice. Pure DAHP synthase obtained was 0.48 mg/mL and at least 90% pure as determined by SDS-PAGE.

3.4.7 Continuous assay of purified DAHP synthase

Purified DAHP synthase was assayed as previously described [20]. Pure DAHP synthase was diluted 4-fold and treated with 50 µM TCEP and 500 µM excess over chelator of each metal in 0.1 M KPi (pH 6.4) anaerobically for 10 minutes at ambient temperature. This mixture was then diluted 1:20 into a 0.5 mL reaction mixture containing saturating amounts of PEP and erythrose 4-phosphate, with or without 100 µM H_2O_2. This sample was assayed by measuring disappearance of the PEP signal at 232 nm over a period of at least 30 minutes. Specific activity obtained from Fe-metallated DAHP synthase was approximately 2.4 ΔA/min/mg protein.

3.4.8 H_2O_2 accumulation

Cells were first grown overnight to stationary phase in anaerobic minimal glucose medium. Cultures were then inoculated to ~ 0.01 OD and grown anaerobically to 0.2 OD. Cells were then used to inoculate fresh aerobic minimal glucose medium to 0.005 OD. Aliquots were removed periodically and filter sterilized before being frozen on dry ice. After all samples were collected, H_2O_2 concentrations were measured by the Amplex

Red/horseradish peroxidase method. Fluorescence was measured using a Shimadzu RF Mini-150 fluorometer.

3.5 TABLES

Strain	Genotype	Source
MG1655	F^- wild-type	*E. coli* Stock Center
LC106	Δ*ahpF* ::*kan* Δ(*katG17* ::Tn*10*)*1* Δ(*katE12* ::Tn*10*)*1*	[56]
JI367	Δ(*katG17* ::Tn*10*)*1* Δ(*katE12* ::Tn*10*)*1*	[54]
AA30	LC106 Δ*mntH2* ::*cat*	[4]
BW25113	*lacI rrnB* Δ*lacZ hsdK* Δ*araBAD* Δ*rhaBAD*	[14]
JS279	BW25113 Δ*tyrR1* ::*cat*	This study
JS282	MG1655 Δ*tyrR1* ::*cat*	This study
SJ130	Δ*lacZ1* with $pINT^{TS}$	Lab stock
JS285	SJ130 *att* λ ::[pSJ501::*aroF' -lacZ* $^+$]	This study
JS289	MG1655 Δ(*tyrR1* ::*cat*)*1*	This study
JS291	MG1655 *att* λ ::[pSJ501::*aroF' -lacZ* $^+$]	This study
JS295	JS289 *att* λ ::[pSJ501::*aroF' -lacZ* $^+$]	This study
JS299	LC106 *att* λ ::[pSJ501::*aroF' -lacZ* $^+$]	This study
BL21 (DE3)	*F* $^-$ *ompT gal dcm lon hsdS* $_B$ (r_B^- m_B^-) λ(DE3)	Novagen
Plasmid	Genotype	Source
$pINT^{TS}$	temperature-sensitive plasmid carrying λ integrase	[28]
pSJ501	pAH125 derivative with *cat* flanked by *frt* sites	Lab stock
pET42a	Expression vector with T7 promoter and N-terminal 8-His tag	Novagen
pAroG-His	pET42a with *aroG* inserted into the NcoI and BamHI sites	This study

Table 3.1: Strain list. These strains and plasmids were used in the DAHP synthase study.

3.6 FIGURES

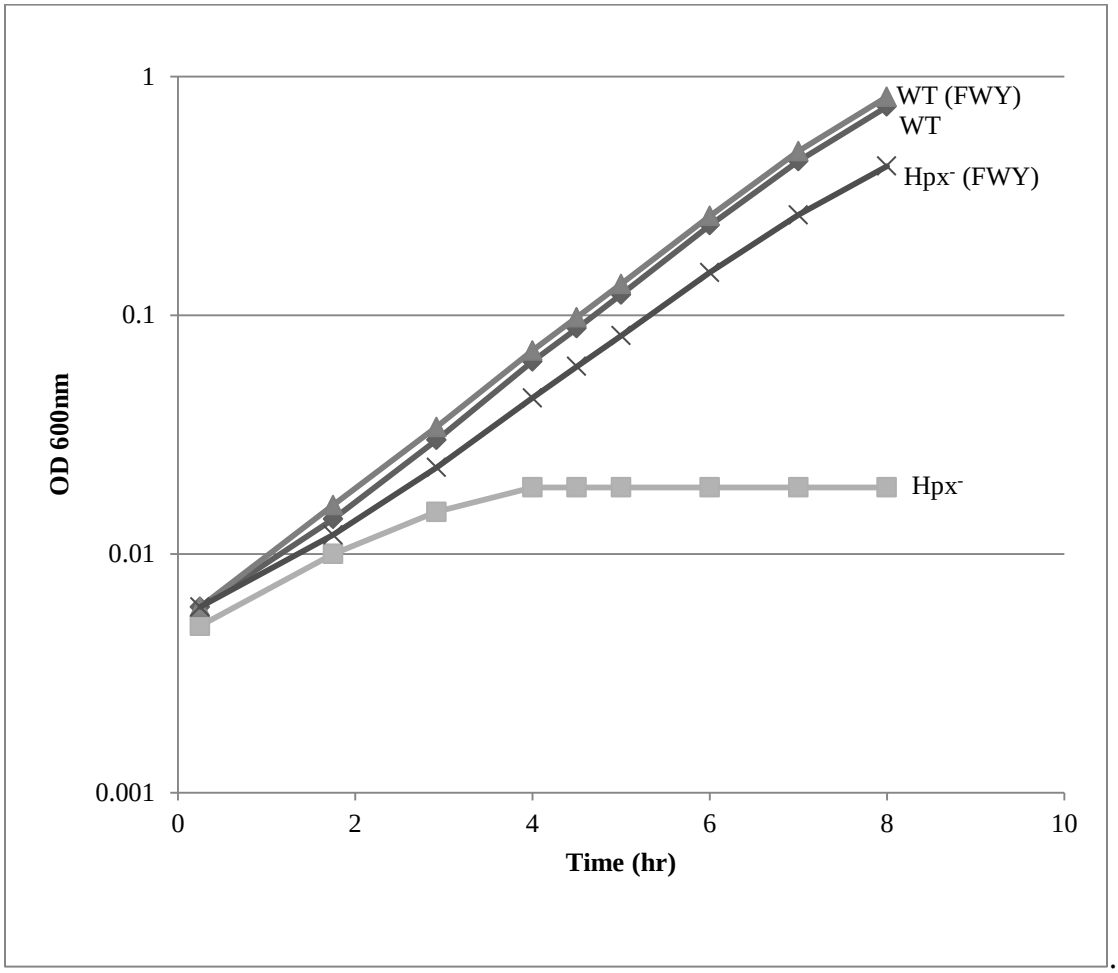

Figure 3.1: Growth of Hpx⁻ cells in minimal A glucose medium. Cultures were grown anaerobically overnight in minimal A glucose medium + thiamin and subcultured anaerobically in the same medium. Cells were diluted into aerobic medium containing or lacking aromatic amino acids at time zero, and growth was monitored aerobically.

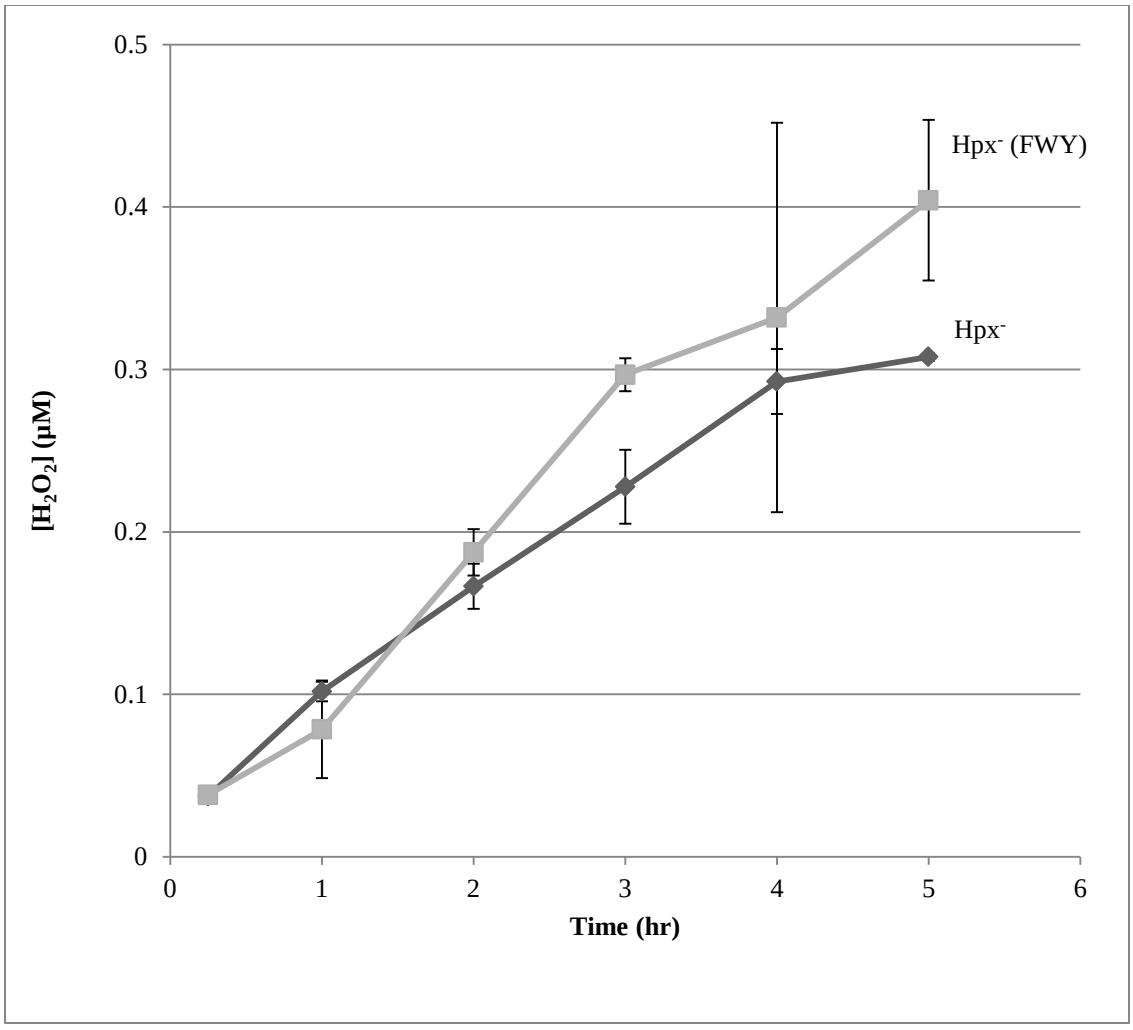

Figure 3.2: Accumulation of H_2O_2 during aerobic growth by Hpx$^-$ cells in minimal A glucose medium. Cultures were grown anaerobically overnight in minimal A glucose medium + thiamin and subcultured anaerobically in the same medium. Cells were diluted into aerobic medium containing or lacking aromatic amino acids at time zero, and growth was monitored aerobically. At times shown, aliquots were removed and the H2O2 concentration of the growth medium was determined by the Amplex Red method. Error bars represent standard deviation from the mean of three independent replicate cultures.

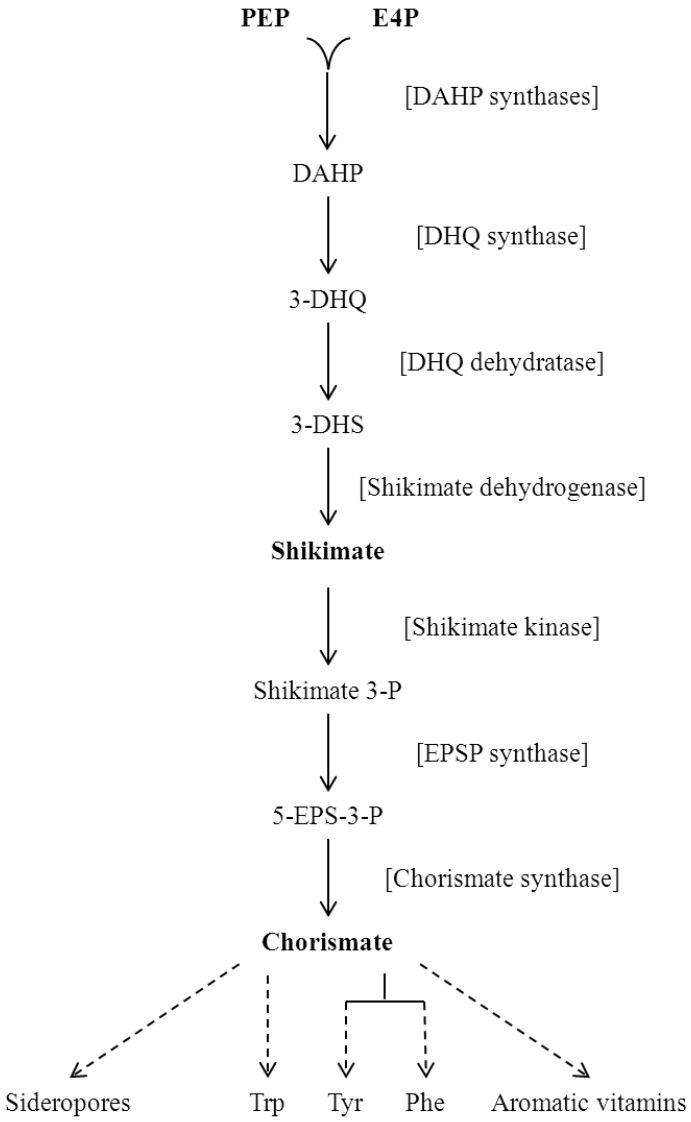

Figure 3.3: Enzymes and intermediates of the shikimate pathway. Solid arrows represent reactions, dashed arrows represent multiple reactions, chemical intermediates are named, and enzymes are denoted in brackets next to the reaction they catalyze. Abbreviations are: phosphoenol pyruvate (PEP), erythrose 4-phosphate (E4P), 3-deoxy-*D-arabino*-heptulosonate 7-phosphate (DAHP), dehydroquinate (DHQ), dehydroshikimate (DHS), 5-Enolpyruvoylshikimate-3-phosphate (5-EPS-3-P), tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe).

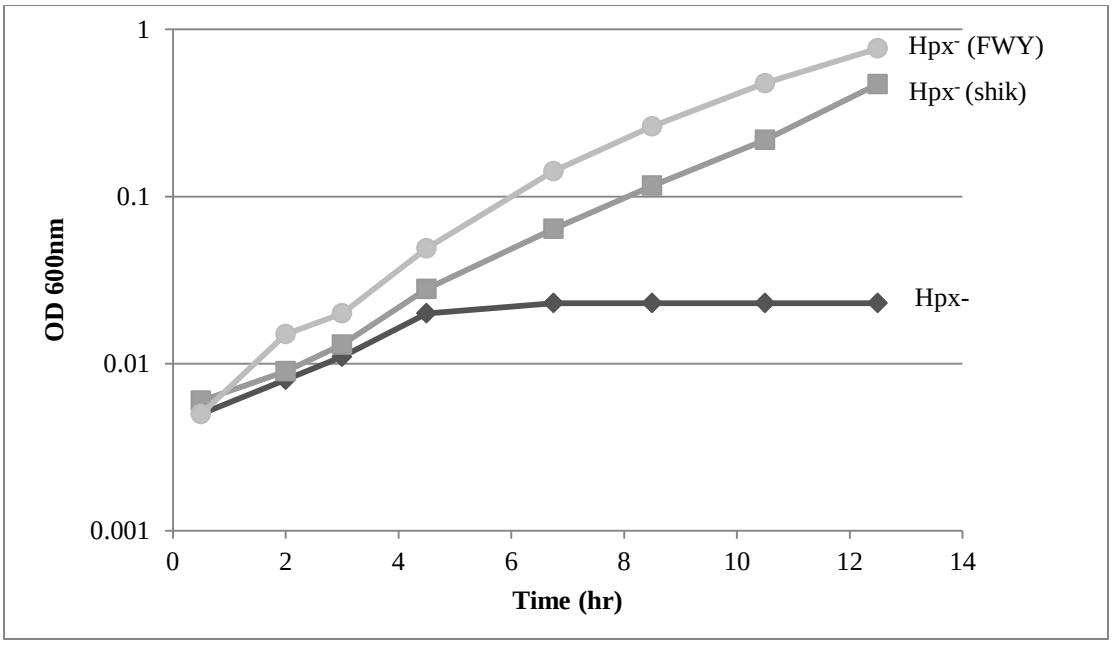

Figure 3.4: Shikimate addition and aerobic growth of Hpx$^-$ cells. Cultures were grown anaerobically overnight in Minimal A Glucose Medium + thiamin and subcultured anaerobically in the same medium. Cells were diluted in the same aerobic medium +/- 50 µM shikimate at time zero, and growth was monitored aerobically.

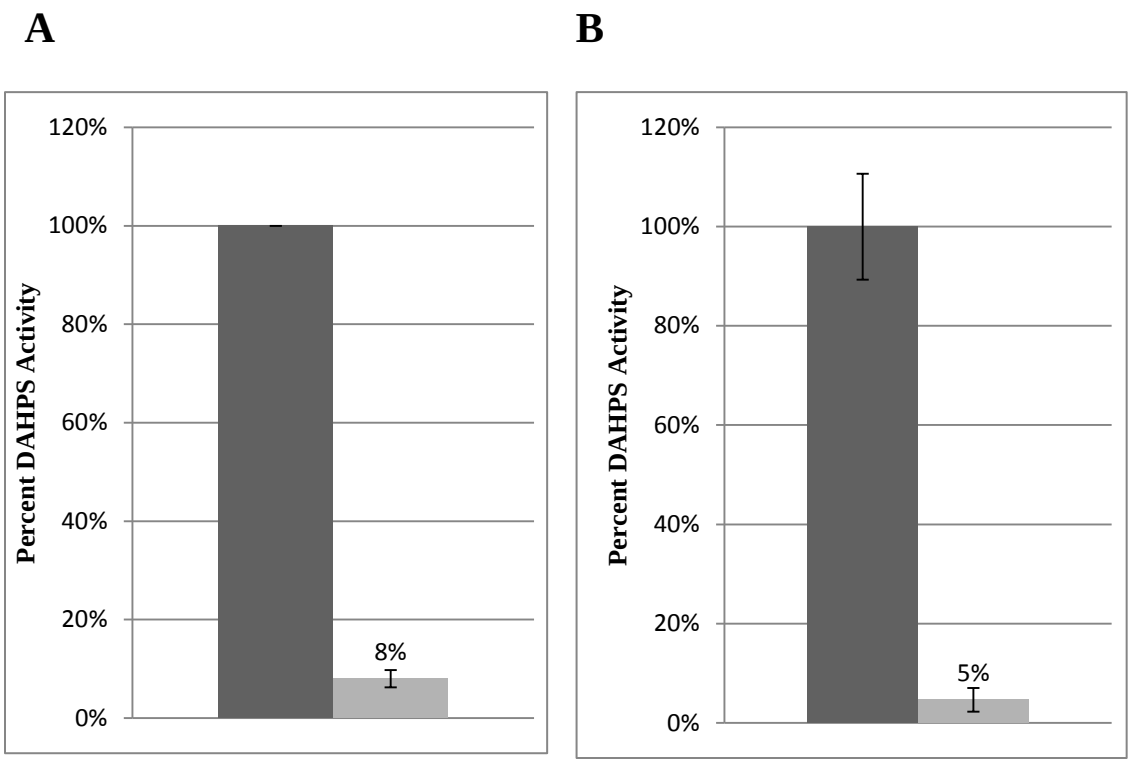

Figure 3.5: DAHP synthase enzyme activity is sensitive to H_2O_2 in vitro. Cultures were grown anaerobically overnight in minimal A glucose medium + thiamin, subcultured **(A)** anaerobically (Hpx^- cells) or **(B)** aerobically (Kat^- cells) in the same medium, and grown to 0.2 OD. Cells were harvested, and extracts were prepared anaerobically in 9 mM PEP + 1 mM DTPA. Samples were challenged anaerobically +/- 100 µM H_2O_2 for 5 minutes (dark bars are without H_2O_2; light bars contain H_2O_2). Reactions were run for 1 min, then stopped by addition of TCA. Determinations of DAHP produced were carried out aerobically. Error bars represent standard deviation from the mean of three independent cultures.

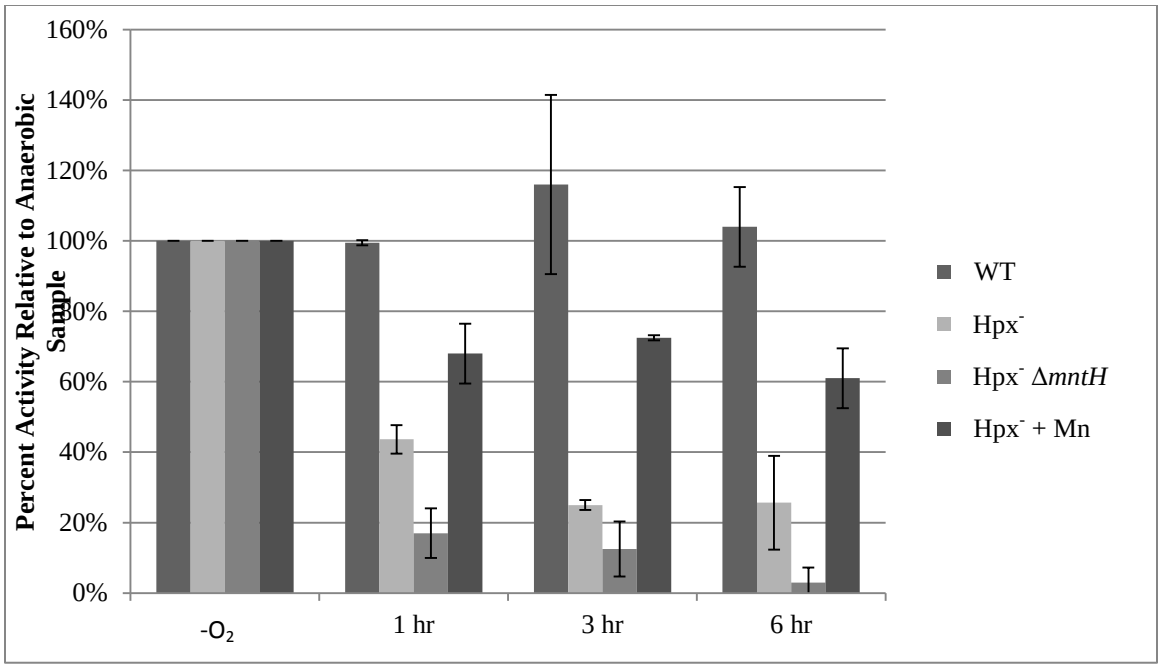

Figure 3.6: DAHP synthase enzyme activity is lost in Hpx$^-$ cells. Cultures were grown anaerobically overnight in minimal A glucose medium + thiamin, subcultured anaerobically in the same medium and grown to 0.2 OD. An anaerobic sample was harvested, and cells were inoculated to 0.05 OD into aerobic medium +/- 1 µM $MnCl_2$, where indicated. Cells were harvested at times noted and extracts were prepared anaerobically in 0.1 M KPi buffer (pH 6.4) containing 9 mM PEP + 1 mM DTPA. Reactions were run for 1 min then stopped by addition of TCA. Error bars represent standard deviation from the mean of three independent cultures.

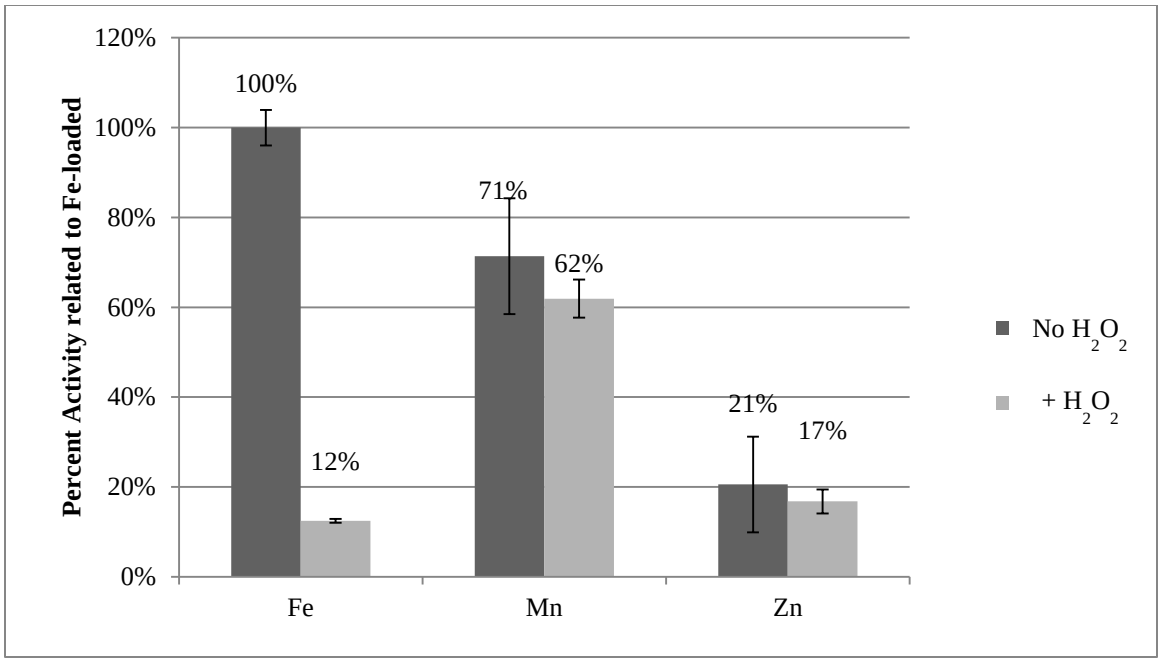

Figure 3.7: Enzyme activity of pure DAHP synthase with various metals. Pure apo-DAHP synthase was treated with 50 µM TCEP and a 500 µM excess of each metal anaerobically for 10 minutes. The enzyme was then diluted 1:20 into a reaction mixture containing saturating amounts of PEP and erythrose 4-phosphate, with or without 100 µM H_2O_2. Activities are given relative to Fe-loaded DAHP synthase without H_2O_2 challenge. Error bars represent standard deviation from the mean of three independent samples.

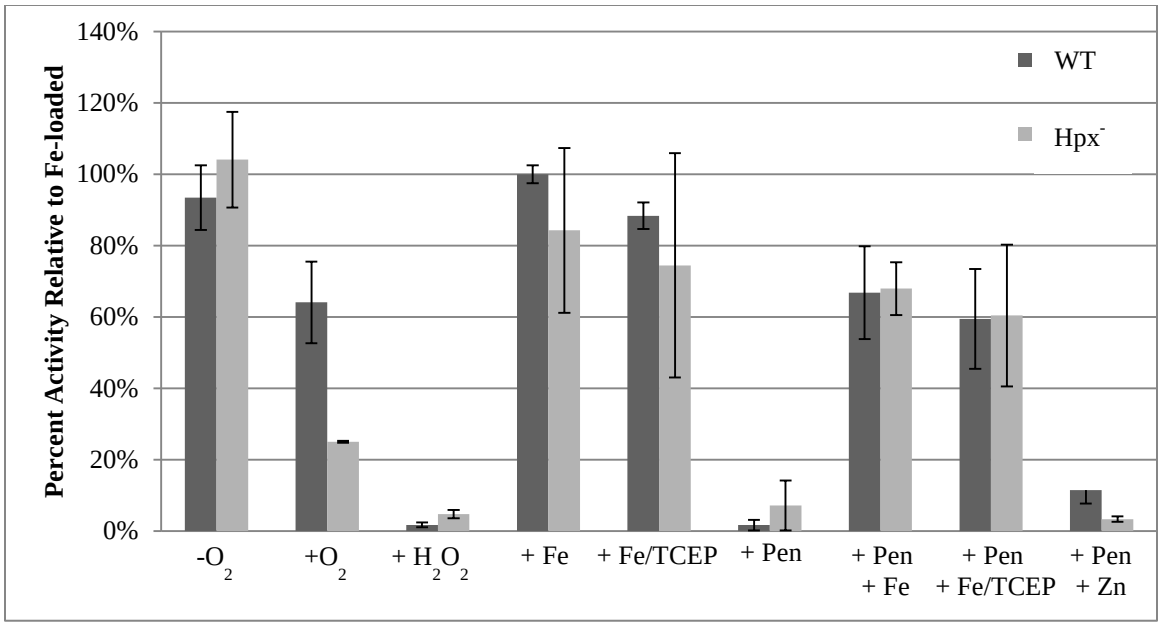

Figure 3.8: DAHP synthase enzyme activity is reactivatable in Hpx⁻ cells. Cultures were grown anaerobically overnight in minimal A glucose medium + thiamin, subcultured anaerobically in the same medium, and grown to 0.2 OD. An anaerobic sample was harvested, and cells were inoculated to 0.05 OD into aerobic medium. Cells were harvested at 0.15 OD and extracts were prepared anaerobically in 9 mM PEP + 1 mM DTPA. Samples were treated with 100 µM H_2O_2 for 5 min, 500 µM Fe, Zn, or Fe/TCEP for 15 min, or 5 mM penicillamine (Pen) for 20 min followed by Zn, Fe, or Fe/TCEP treatments as noted above. Reactions were run for 1 min, then stopped by addition of TCA. Error bars represent standard deviation from the mean of three independent cultures.

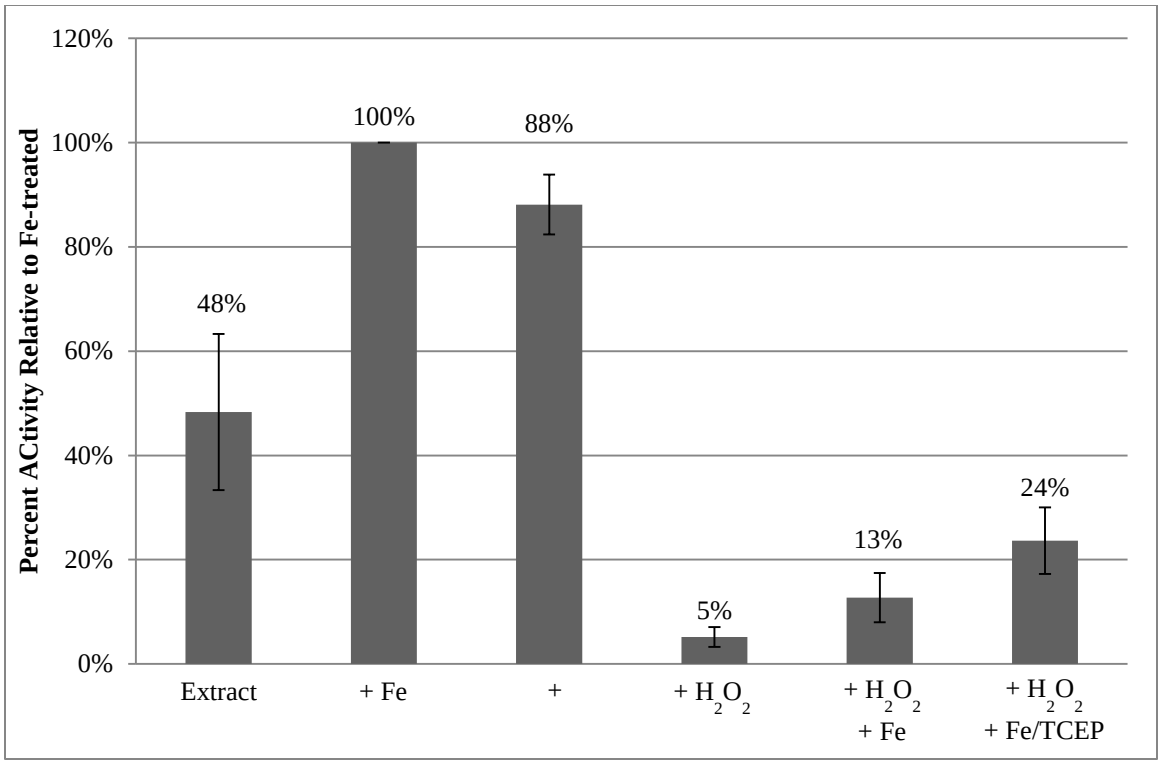

Figure 3.9: DAHP synthase enzyme activity is only partially reactivatable after in vitro damage. Cultures were grown anaerobically overnight in minimal A glucose medium + thiamin, subcultured anaerobically in the same medium, and grown to 0.2 OD. Cells were harvested and extracts were prepared anaerobically in 9 mM PEP + 1 mM DTPA. Where indicated, samples were treated with H_2O_2 (100 µM for 5 min), 500 µM Fe or Fe/TCEP for 15 min. Reactions were run for 1 min and then stopped by addition of TCA. Error bars represent standard deviation from the mean of three independent cultures.

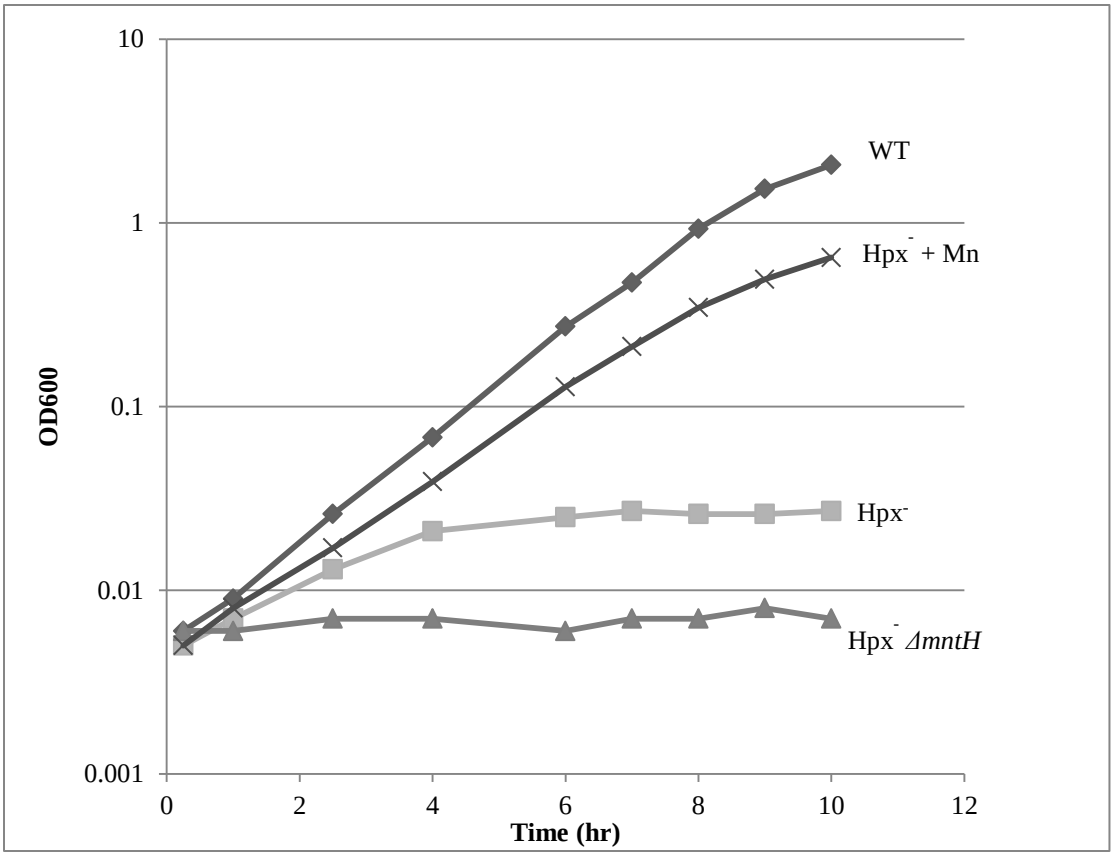

Figure 3.10: Manganese addition and aerobic growth of Hpx- cells. Cultures were grown anaerobically overnight in Minimal A Glucose Medium + thiamin and subcultured anaerobically in the same medium. Cells were diluted in the same aerobic medium +/- 50 µM Mn at time zero, and growth was monitored aerobically.

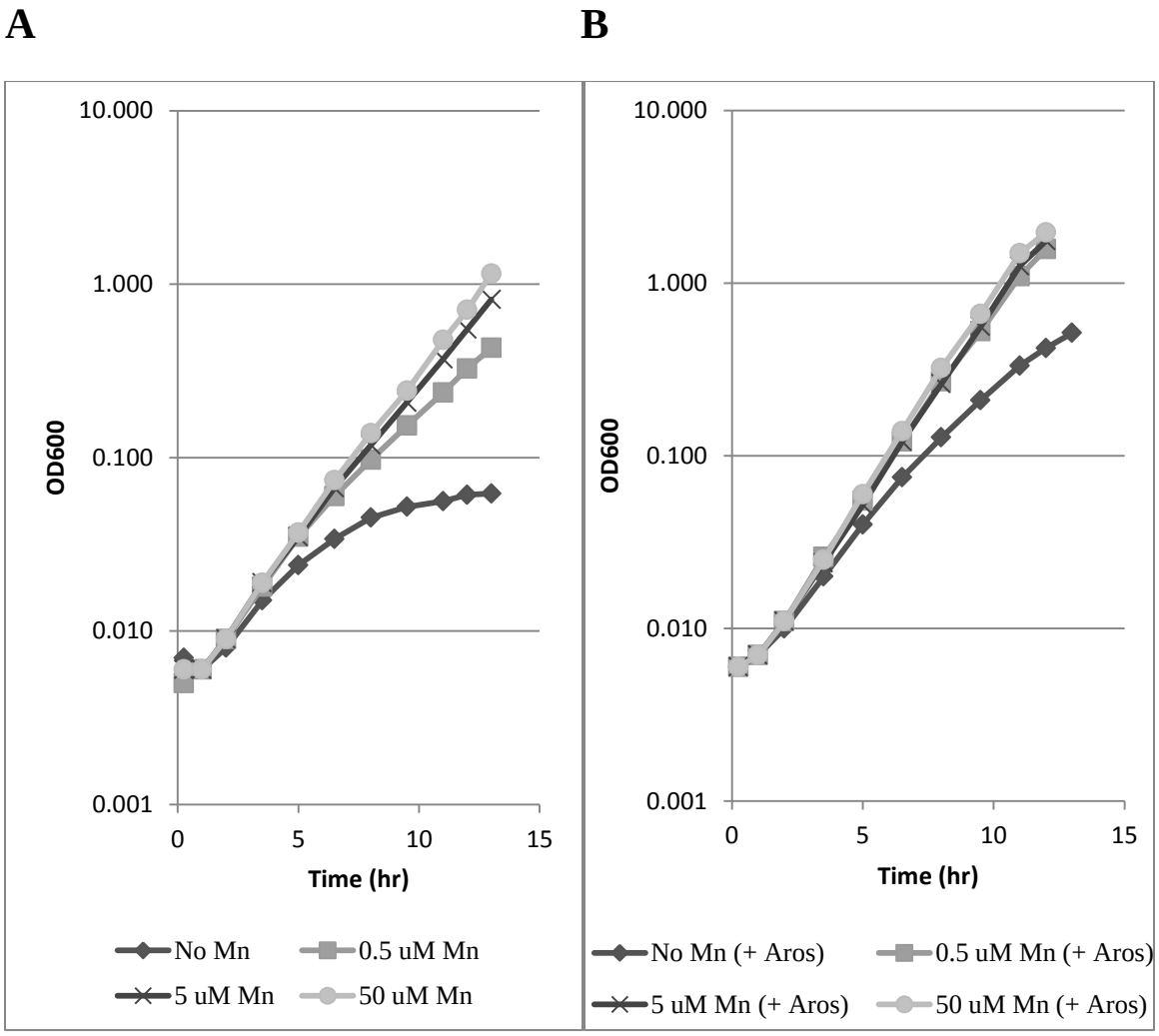

Figure 3.11: Manganese addition and aerobic growth of Hpx⁻ cells. Cultures were grown anaerobically overnight in Minimal A Glucose Medium + thiamin and subcultured anaerobically in the same medium. Cells were diluted in the same aerobic medium with **(A)** or without **(B)** aromatic amino acids +/- Mn as indicated at time zero, and growth was monitored aerobically.

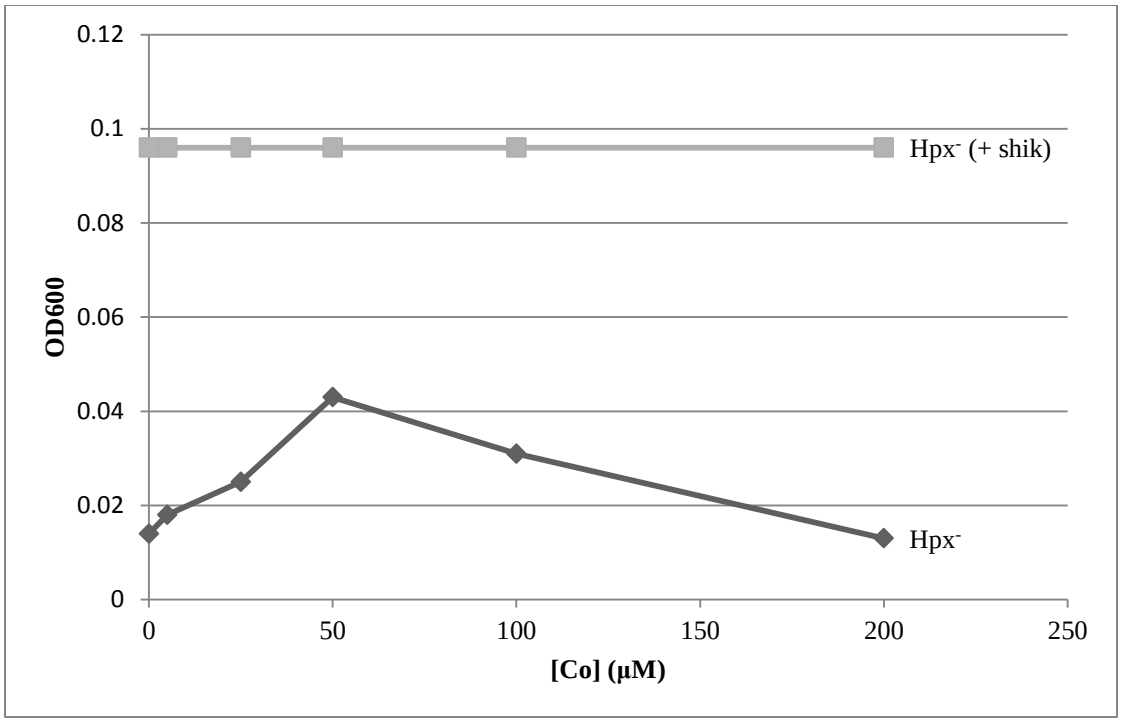

Figure 3.12: Cobalt addition and aerobic growth of Hpx$^-$ cells. Cultures were grown anaerobically overnight in Minimal A Glucose Medium + thiamin and subcultured anaerobically in the same medium. Cells were diluted in the same aerobic medium +/- Co or 50 mM shikimate (shik) as indicated at time zero, and growth was monitored aerobically. Points represent the OD measured after 10 hours of aeration.

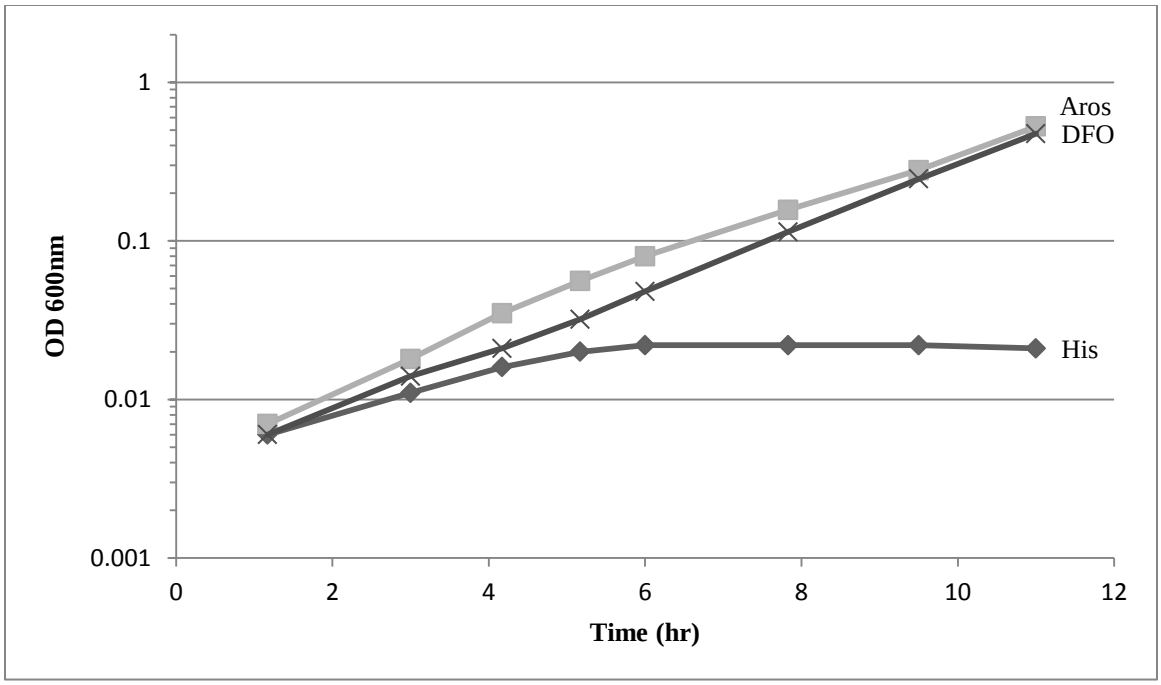

Figure 3.13: Deferroxamine addition and aerobic growth of Hpx⁻ cells. Cultures were grown anaerobically overnight in Minimal A Glucose Medium + thiamin and subcultured anaerobically in the same medium. Cells were diluted in the same aerobic medium +/- 1 mM deferoxamine (DFO) at time zero, and growth was monitored aerobically.

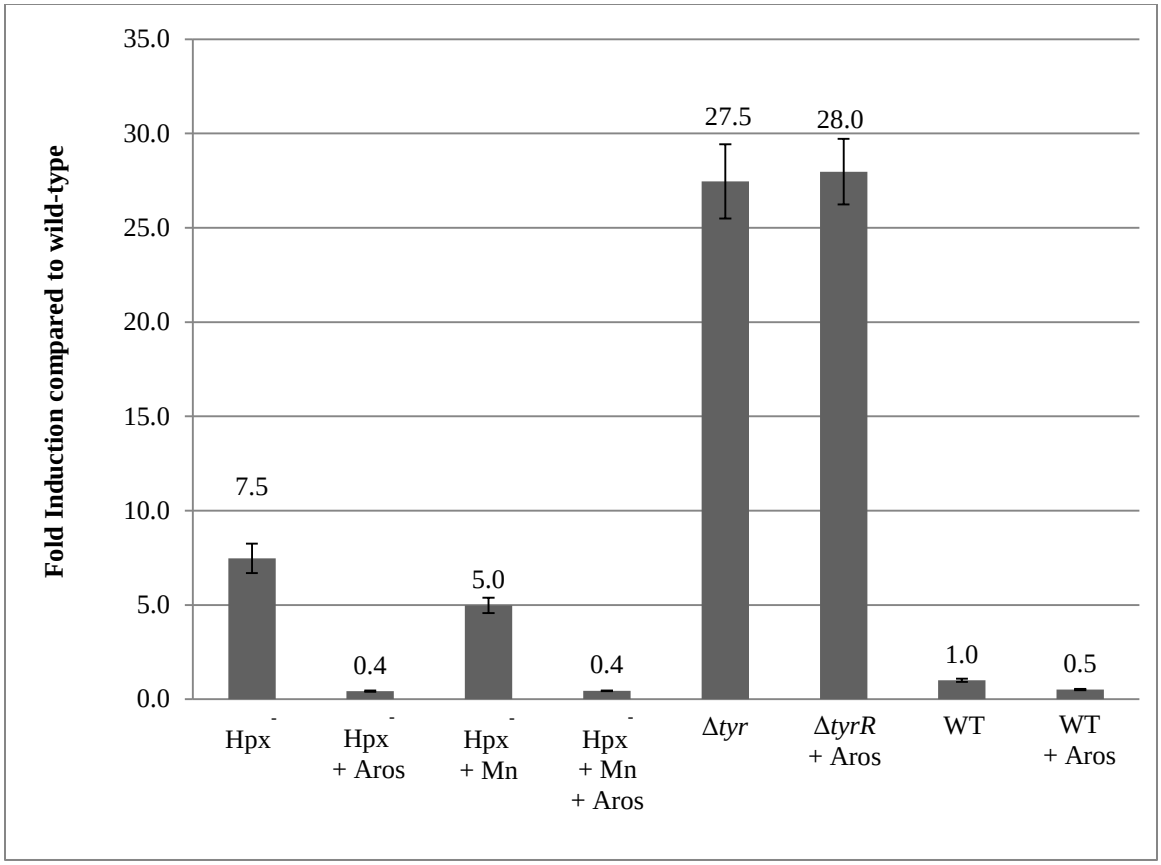

Figure 3.14: DAHP synthase transcription increases in response to H_2O_2 stress. Cultures were grown anaerobically overnight in minimal A glucose medium + thiamin, subcultured anaerobically in the same medium, and grown to 0.15 OD. Cells were then inoculated into fresh aerobic medium +/- 50 mM aromatic amino acids (Aros) or +/- 50 mM Mn, where indicated, and grown aerobically for 4 hours. Cell extracts were prepared, and β-galactosidase activity from a P*aroF*'-*lacZ* transcriptional fusion integrated into the λ attachment site was determined [50]. Error bars represent standard deviation from the mean of three independent cultures.

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CHAPTER 4: CONCLUSIONS

4.1 SUMMARY OF CURRENT WORK

4.1.1 Mononuclear iron enzymes are primary targets of H_2O_2

Under normal growth conditions, *Escherichia coli* produces low amounts of H_2O_2 as an inadvertent byproduct of aerobic metabolism. When its H_2O_2 scavengers, catalase and peroxidase, are deleted from the cell, the resultant Hpx^- strain exhibits a number of phenotypes. The first phenotype discovered, damage to DNA, was found to be due to hydroxyl radical oxidation of the DNA backbone as a result of Fenton chemistry occurring on the DNA [18]. Other phenotypes, including an inability to grow on TCA cycle intermediates, failure of the Isc system, and a leucine auxotrophy, were all due to Fenton chemistry leading to oxidation of solvent-exposed [4Fe-4S] cluster dehydratases [14, 15]. In this study, two other phenotypes were explored.

The first phenotype I examined was a block in the pentose phosphate pathway. I found that when Hpx^- *E. coli* cells were forced to metabolize gluconate through the pentose phosphate pathway, they were unable to grow more than one or two doublings in air. I measured the activity of each of these five enzymes downstream of gluconate in the pentose phosphate pathway and found that only one, Rpe, lost activity upon H_2O_2 exposure. This result was interesting for a number of reasons. First, no one had ever observed damage to proteins other than [4Fe-4S] cluster dehydratases by H_2O_2, making Rpe a completely novel protein target. Second, Rpe was not known to use iron as a cofactor [1]. This result meant either that Rpe must actually use iron, although other researchers found it was unable to be activated by iron, or that damage to Rpe was not Fenton-based. All other known forms of H_2O_2 damage are mediated by the Fenton reaction [2, 14, 15, 17, 18, 21], so the idea that Rpe was somehow an exception did not seem likely. When Rpe was purified and tested anaerobically, I found that not only was iron able to activate it, but that iron activation yielded the highest amount of activity of

any metal. In addition, iron was the only metal that conferred H_2O_2 sensitivity, which meant that iron was the physiological metal for Rpe. Thus, damage to Rpe mirrors that of the [4Fe-4S] cluster dehydratases, as both use the metal to bind and activate substrate. It is not a surprise, then, that these proteins have similar inactivation rates, about $4 – 5 \times 10^3$ M^{-1} s^{-1}. Once again, Fenton chemistry is the reason for damage by H_2O_2.

There is another important aspect of this finding to note beyond the sensitivity of Rpe to H_2O_2. By performing the metallation reaction in the anaerobic chamber, I was able to maintain iron in the ferrous form, while researchers working with the protein under aerobic conditions were unable to keep the iron reduced. It is conceivable that this experimental artifact could have misled a number of researchers performing metallation studies on mononuclear metalloproteins. Adil Anjem tested three other such proteins and found that all were activated by iron and sensitive to H_2O_2, in vitro and in vivo, showing that these proteins also used iron as their physiological cofactor [2]. Thus, the mononuclear iron protein class may continue to grow as more work is done to determine the metals used by various proteins.

The second phenotype I explored was the aromatic amino acid auxotrophy. When work first began on this phenotype, it was not known that mononuclear iron proteins were targets of H_2O_2. Since the aromatic amino acid biosynthetic pathway contained no Fe-S cluster proteins, no protein seemed more likely to be damaged than any other. In fact, there was reason to believe that transketolase was the cause, as this would also explain the problem with the pentose phosphate pathway. However, when the sensitivity of Rpe was found, two problems were solved. First, the two phenotypes were separated from one another, meaning that another target existed. Second, identification of the mononuclear iron proteins as a class of H_2O_2 targets shed new light on potential targets in the shikimic acid pathway for aromatic biosynthesis. I identified the first enzyme in the pathway, DAHP synthase, as a mononuclear iron protein and found that it, like the others in this class, was sensitive to H_2O_2.

4.1.2 Mechanism of protection of mononuclear iron proteins by manganese

Manganese has long been known to have protective effects during oxidative stress. Manganese was initially believed to protect cells by scavenging reactive oxygen species; however, this mechanism of protection was shown to be incorrect in *E. coli*, as scavenging rates did not change when manganese was added to cells [3]. In this study, I sought to show that manganese protection, at least in part, is due to metallation of mononuclear iron proteins. While metallation by iron causes these proteins to be sensitive to H_2O_2, metallation by manganese provides a moderate amount of activity that is not sensitive to oxidation by H_2O_2. I showed that manganese is able to protect two mononuclear iron enzymes, Rpe and DAHP synthase, from oxidation by H_2O_2 in vivo and in vitro. As mentioned previously, I believe that mononuclear iron proteins are much more common than is currently known, so the protective effect of manganese on this class of proteins is an important factor in combatting the negative effects of reactive oxygen species. *E. coli* recognizes the importance of manganese during oxidative stress, as evidenced by the upregulation of the manganese transporter, MntH, by OxyR. Without MntH, Hpx^- cells are unable to grow, even with amino acid supplements, highlighting the importance of manganese during H_2O_2 stress.

4.2 POSSIBLE FUTURE DIRECTIONS

4.2.1 Why does *E. coli* require iron in a pathway needed to acquire it?

One additional problem that damage to DAHP synthase causes for *E. coli* is limitation of its ability to import iron. In fact, there are at least two other proteins in the pathway that might also use iron but are not annotated as such. While this might not be such a problem during oxidative stress, as iron limitation is one of the desired outcomes of induction of the stress responses, it is more difficult to comprehend why *E. coli* would require iron for a pathway that produces the precursor for synthesis of enterochelin, a siderophore used to acquire iron from the environment. Preliminary results of

enterochelin synthesis during H_2O_2 stress indicate that damage to DAHP synthase results in an inability to make siderophores (Figure 4.1). It is inconceivable that iron levels should fall so low that there is no DAHP synthase activity, but there are seven different metabolites from five distinct pathways that require chorismate, the end product of the shikimic acid pathway for their synthesis [10]. Perhaps the answer as to how *E. coli* manages this problem comes from an investigation of the K_M for chorismate of the branch pathways. The pathway with the lowest K_M, somewhat surprisingly, is for tryptophan synthesis [7]. The pathways for folate and ubiquinone biosynthesis have the next lowest K_M values for chorismate [20, 22]. It is easy to see why these pathways might be given preference over other metabolites, as they are key to keeping energy levels high enough to keep metabolism running. Next in the chorismate hierarchy is synthesis of enterochelin. This pathway has a K_M for chorismate of 14 μM [16], more than three times lower than the next processes, which are the pathways for phenylalanine [9] and tyrosine [13] biosynthesis and at 195 μM, the pathway for menaquinone biosynthesis [8]. This analysis of apparent K_M values indicates the importance of the ability of the cell to synthesize these iron importers, greater even than synthesis of two amino acids. Further exploration of the pathway and its activity during iron starvation may provide insight into how *E. coli* prioritizes iron, as well as flux through this pathway.

4.2.2 Are mononuclear iron proteins mismetallated during H_2O_2 stress, and if not, how does the cell prevent it?

While I showed that DAHP synthase does not become mismetallated by zinc during oxidative stress, other mononuclear iron proteins have not been so rigorously tested for this phenotype. In fact, there is evidence that SOD mutants lose activity of mononuclear iron proteins first by oxidation of the iron atom, but that activity remains low because the iron is replaced by zinc [12]. It is possible that during H_2O_2 stress, manganese import by induction of MntH shifts the metal balance in the cell to favor metallation by manganese over iron or zinc, but in SOD mutants, there is no induction of manganese import. Therefore, during $O_2^{\cdot -}$ stress, no metals are available to compete with

zinc for the active sites of mononuclear iron proteins, resulting in accumulation of inactive zinc-metallated protein.

4.2.3 Are there other mononuclear iron proteins that are sensitive to H_2O_2?

As work continues to identify additional phenotypes of H_2O_2 stress, more mononuclear iron proteins may be identified that cause them. Ongoing work in the lab is using RNA-Seq to identify genes that are upregulated in response to oxidative stress. While some genes – notably, the members of the OxyR regulon – are expected to appear, other genes whose products are sensitive to H_2O_2 may also be induced. DAHP synthase is an example of this, as *E. coli* attempts to produce more protein in response to the decreased activity of the pathway. Additionally, proteins that use metal cofactors for non-redox reactions may be additional targets. While some of the novelty has been lost, it remains important to identify additional targets of H_2O_2 in order to fully appreciate the scope of the effects reactive oxygen species have on cells.

4.2.4 Are mononuclear iron proteins sensitive to H_2O_2 in organisms that either do not use iron or produce H_2O_2?

Some bacteria, such as *Borrelia Burgdorferi* and *Lactococcus plantarum*, are considered to be low-iron or even iron-free organisms that import little to no iron [4, 5, 11, 19]. These organisms, presumably as a way to compensate for a lack of iron, import high levels of manganese. It is possible that these, and organisms like them, have evolved to use manganese in the place of iron in mononuclear metalloproteins. Use of manganese in these proteins may be one explanation for the ability of lactic acid bacteria to survive under conditions where they are producing up to millimolar levels of H_2O_2.

One way to examine this possibility is to examine *Lactococcus* counterparts of the proteins found to be mononuclear iron enzymes in *E. coli*. If these proteins are found to be sensitive to H_2O_2 as well, it will be interesting to determine what metal is being used in these proteins, since the organism is not known to import iron. Alternatively, if the proteins are resistant and found to bind manganese, it would be beneficial to determine if

there are differences that might allow the protein to differentiate between iron and manganese. This could be tested both by expressing the *Lactococcus* proteins in *E. coli* to see if they become sensitive, and conversely by expressing the *E. coli* proteins in *Lactococcus* to see if they are now resistant to H_2O_2.

4.2.5 Have eukaryotes evolved to use alternative metals in mononuclear metalloproteins to protect against oxidative stress?

It is possible that eukaryotes also suffer from the same damage to mononuclear iron enzymes experienced by *E. coli* cells as a result of oxidative stress. It would be interesting to determine whether damage to any of these proteins in humans is responsible for any known diseases. It is also likely, however, that eukaryotic organisms have taken evolutionary steps to rid themselves of this vulnerability. Preliminary results using Baker's yeast suggest that this may be the case, as Rpe assayed from aerobically grown *Saccharomyces cerevisiae* showed no inactivation by 1 mM H_2O_2 and is described fully in Appendix B. This result suggests either that yeast have evolved to use a metal other than iron in the active site of Rpe, or that yeast Rpe somehow shields its metal from H_2O_2. Further work must be carried out to determine the mechanism of resistance of yeast Rpe to H_2O_2; such work might include experiments such as expression of yeast Rpe in *E. coli* and *vice versa*. Other mononuclear iron proteins should also be examined to determine whether this resistance in yeast is specific to Rpe or extends to mononuclear iron proteins in general.

4.3 FIGURES

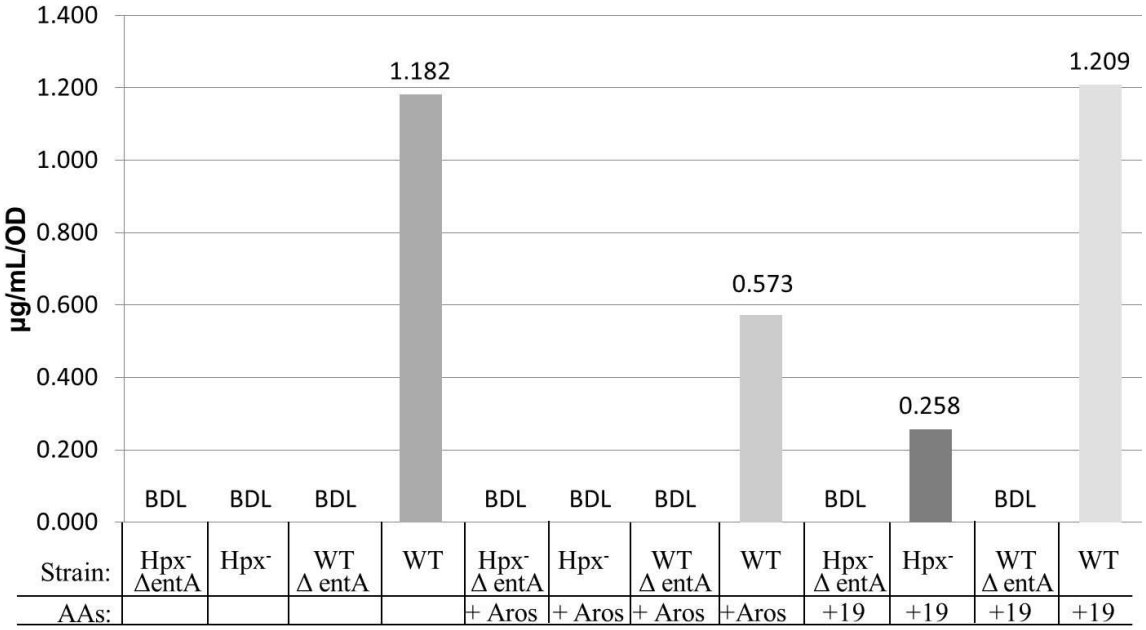

Figure 4.1: Enterochelin synthesis of Hpx⁻ cells. Cultures were grown anaerobically overnight in Minimal A Glucose Medium + thiamin and subcultured anaerobically in the same medium. Cells were diluted in the same aerobic medium +/- aromatic or 19 amino acids as indicated at time zero and cells were grown overnight. Cells were then spun out and the supernate was used to assay for catechol by the method of Arnow [6]. (BDL = below detectable limit.)

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APPENDIX A: DETERMINATION OF FREE IRON LEVELS IN RESPONSE TO RYHB IN *ESCHERICHIA COLI*

A.1 INTRODUCTION

RyhB is a non-coding small RNA in *Escherichia coli* that was discovered by Massé and Gottesman in 2002 [2]. RyhB is repressed by metallated Fur protein, but when ferrous iron levels are low and the Fur regulon is derepressed, RyhB is expressed. A global transcription microarray analysis using a plasmid expressing RyhB showed it was able to directly repress at least 18 transcripts, including many genes that require iron for activity [3]. It has been suggested that the purpose of RyhB is to spare iron from dispensable iron proteins in order to allow metallation of indispensable proteins that require it [1]. Because previous work was performed in complex medium, which has a high level of iron, it was difficult to see distinct phenotypes of RyhB deletion. This study provides evidence that RyhB spares iron in media where iron levels are very low.

A.2 RESULTS

A.2.1 RyhB mutants display a growth phenotype as they reach stationary phase

While rich media, such as LB, are replete with iron, minimal media contain low levels of iron unless it is supplemented. Therefore, when looking for growth phenotypes related to iron homeostasis, minimal defined media allow for the elucidation of growth phenotypes that would not be apparent in rich medium. For this reason, strains obtained from the Eric Masse lab – EM1055 (wild-type), EM1238 (Δ*ryhB*), EM1256 (Δ*fur*), and EM1257 (Δ*ryhB*, Δ*fur*) – were grown in M63 glucose medium. While there was no marked difference in the growth of these strains during logarithmic growth, as the cells approached stationary phase, strains containing the Δ*ryhB* allele were unable to reach as

high an optical density as strains that were wild-type for RyhB (Figure A.1A). However, when 1 μM $FeSO_4$ was added to the growth medium, this growth phenotype was abolished (Figure A.1B). Thus, RyhB either is required to provide iron for some process that becomes important during stationary phase, or is needed to allow proper distribution of iron when it becomes scarce.

A.2.2 RyhB mutants do not accumulate high levels of intracellular free iron

In order to examine whether RyhB was able to affect intracellular levels of iron in minimal medium, electron paramagnetic resonance (EPR) spectroscopy was performed. EPR provides a quantitative measure of chelatable iron levels inside the cell, which, by extension, is a measure of the free and available iron pools in the cell. If RyhB were sparing iron for indispensable processes that require it, then a RhyB mutant should have lower levels of free iron than a wild-type cell under conditions where iron is scarce. Bars 1 and 3 of Figure A.2 show that this is indeed true. Even when iron is added to the medium, the RyhB mutant strain does not accumulate high levels of free iron (Figure A.2, bar 4). This is not the case for the *fur* mutant, which accumulates 4.5-fold more iron than the wild-type strain when no iron was added (Figure A.2, bar 5) or more than 16-fold more when iron was added to the growth medium (Figure A.2, bar 6). This is not surprising, as deletion of fur will lead to constitutive expression of iron import systems, as well as iron-sparing by RyhB. Interestingly, when the *ryhB* and *fur* deletions were combined, the increase of intracellular free iron seen in the *fur* mutant was completely abolished (Figure A.2, bars 7 and 8). This suggests that iron sparing by RyhB is what allows the *fur* mutants to accumulate so much free iron.

A.3 DISCUSSION

Determination of the role of RyhB in iron homeostasis is integral to acquiring a full understanding of how iron pools are regulated during changing environmental conditions. RyhB has been shown to be involved in iron sparing [1] in rich medium.

This report both verifies that this is true of minimal medium, but also demonstrates a growth phenotype as cells approach stationary phase. It is important to note that this medium was not treated to remove trace amounts of iron that might contaminate the various components of the medium. Therefore, if iron levels in the medium were more limited, the growth lag observed as cells reach stationary phase may occur earlier as iron pools are exhausted.

Taken together, the growth phenotype and the free iron levels seem to agree with the idea that RyhB spares the cell iron, which is detected as free iron. Failure to spare the iron (under iron-limited conditions) causes the cells to stop growing, however the specific defect that was responsible for the growth defect was not identified. Thus, RhyB is an integral member of the Fur regulon whose importance to the cell during conditions that limit the availability of iron warrants further study.

A.4 EXPERIMENTAL PROCEDURES

A.4.1 Reagents

Diethylenetriaminepentaacetic acid (DTPA), desferrioxamine mesylate, and $FeCl_3$ hexahydrate were from Sigma, while $FeSO_4$ was from Bioshop. All other chemicals used were from Fisher.

A.4.2 Bacterial growth

Cells were grown overnight in M63 medium containing 0.2% glucose in the absence or in the presence of 1 μM $FeSO_4$. Overnight cultures were then used to inoculate 15 mL same medium to 0.14 OD and growth was monitored at 37°C.

A.4.3 EPR analysis

A.4.3.1 Growth

Cells were grown overnight at 37°C in M63 glucose medium, diluted 1:10 into 250 mL freshly prepared M63 glucose +/- 1 µM $FeSO_4$, and then grown at 37°C in 1L baffled flasks with vigorous shaking to a cell density of approximately 0.9 OD at 600 nm. Cells were then harvested and prepared for EPR analysis.

A.4.3.2 Sample preparation

Cultures were placed on ice for 10 minutes, and then spun for 10 minutes at 6,000 rpm at 4°C in 250 mL bottles using the Beckman JA-14 rotor. Pellets were resuspended in 8 mL M63 glucose + 1 mL 0.1 M DTPA (pH 7.0) that had been pre-warmed to 37°C. Next, 1 mL 0.2 M DFO (pH 8.0) was added and the culture was shaken for 15 minutes at 37°C.

Samples were then put on ice for 15 minutes before being spun for 5 minutes at 8,000 rpm at 4°C. Supernatants were discarded and pellets were washed twice with 5 mL ice-cold 20 mM Tris-HCl (pH 7.5), spinning for 5 minutes at 8,000 rpm at 4°C after each wash. Finally, pellets were resuspended in 300 µL ice-cold 20 mM Tris-HCl (pH 7.4) containing 30% glycerol. About 200 µL of each of the final suspensions was added to separate EPR tubes, shaken to the bottom of the tube, and frozen immediately on dry ice.

Optical densities of the samples were measured for use in determination of intracellular volumes. To do this, 10 µL of each sample was diluted into 990 µL 20 mM Tris-HCl (pH 7.4) containing 30% glycerol and mixed well. Each of these was then diluted 1:10 into the same buffer and the OD was measured. Three individual OD replicates were measured for each sample.

A.4.3.3 Preparation of Fe standards

A fresh stock of 10 mM $FeCl_3 \cdot 6H_2O$ was prepared by adding 0.27g $FeCl_3 \cdot 6H_2O$ to 10 mL of 20 mM Tris-Cl (pH 7.4) + 10% glycerol. Dilutions were then prepared in the same buffer to theoretical concentrations from 0 to 100 µM Fe. Concentrations of the

standards were then determined by measuring the A_{420} with the extinction coefficient of 2865 $M^{-1}cm^{-1}$. About 200 µL of each standard was added to separate EPR tubes for generation of a standard curve.

A.4.3.4 EPR spectrometer parameters

Field controller:

Field set: 01573 (g = 4.3)

Scan source: Ext

Scan range (field sweep): 4 x 100

Function selector:

High frequency

Cavity Modulation: I: High II: Low

Gain: 3.2 x 103 Norm

Power: 10 mW

Norm: 180

Modulation: 1.25 x 10

Function: 100 kHz

Time Constant: 0.032

Field Sweep: Off

Computer:

Scans: 7 scans; 30 sec/scan; 3 sec between scans

A.5 FIGURES

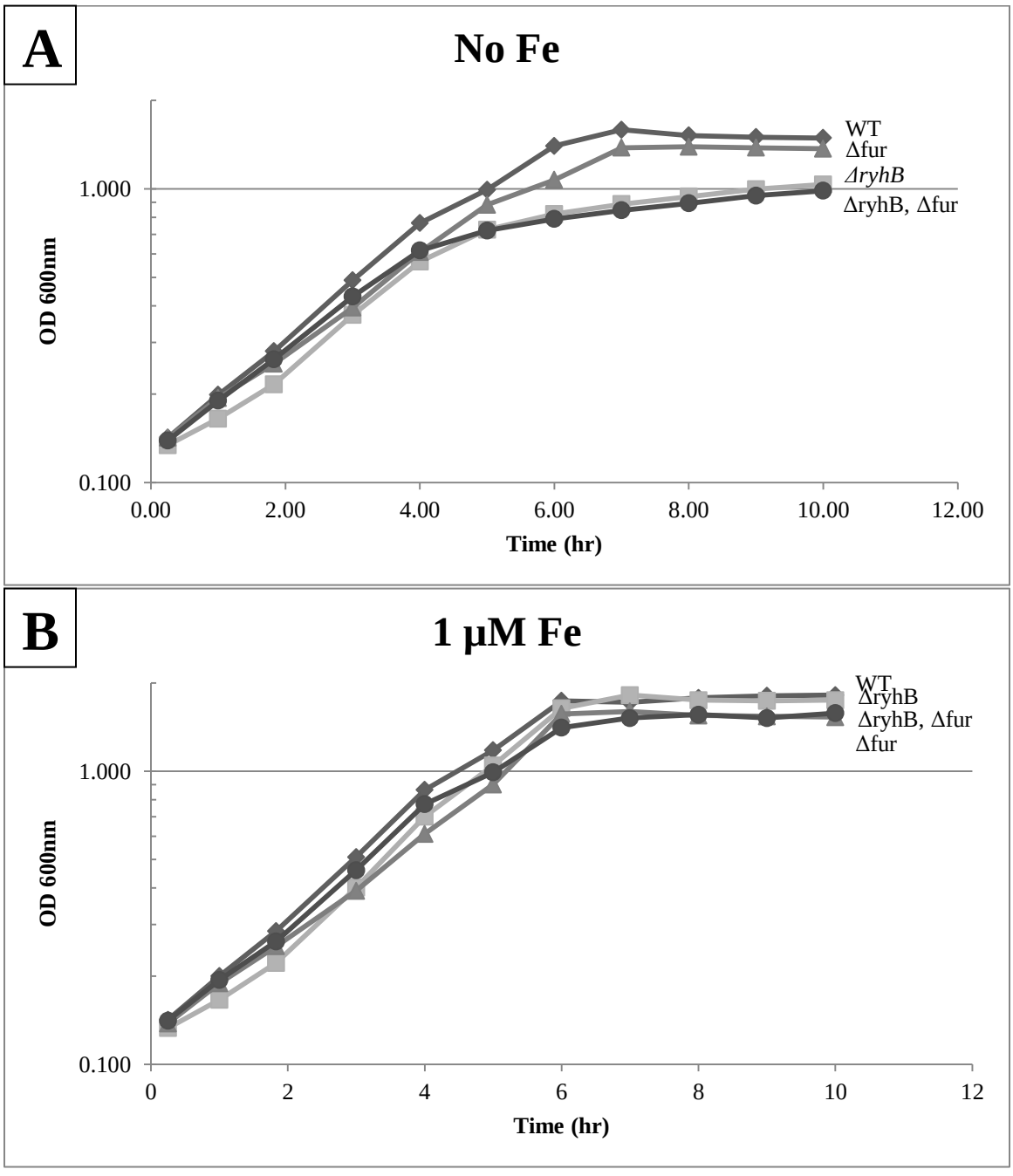

Figure A.1: Growth of ryhB mutants is impaired near stationary phase in minimal medium. Cells were grown overnight in M63 medium containing 0.2% glucose in the absence (A) or in the presence (B) of 1 µM $FeSO_4$. Overnight cultures were then used to inoculate 15 mL same medium to 0.14 OD and growth was monitored at 37°C. Strains used were EM1055 (WT), EM1238 (Δ*ryhB*), EM1256(Δ*fur*), and EM1257(Δ*ryhB*, Δ*fur*).

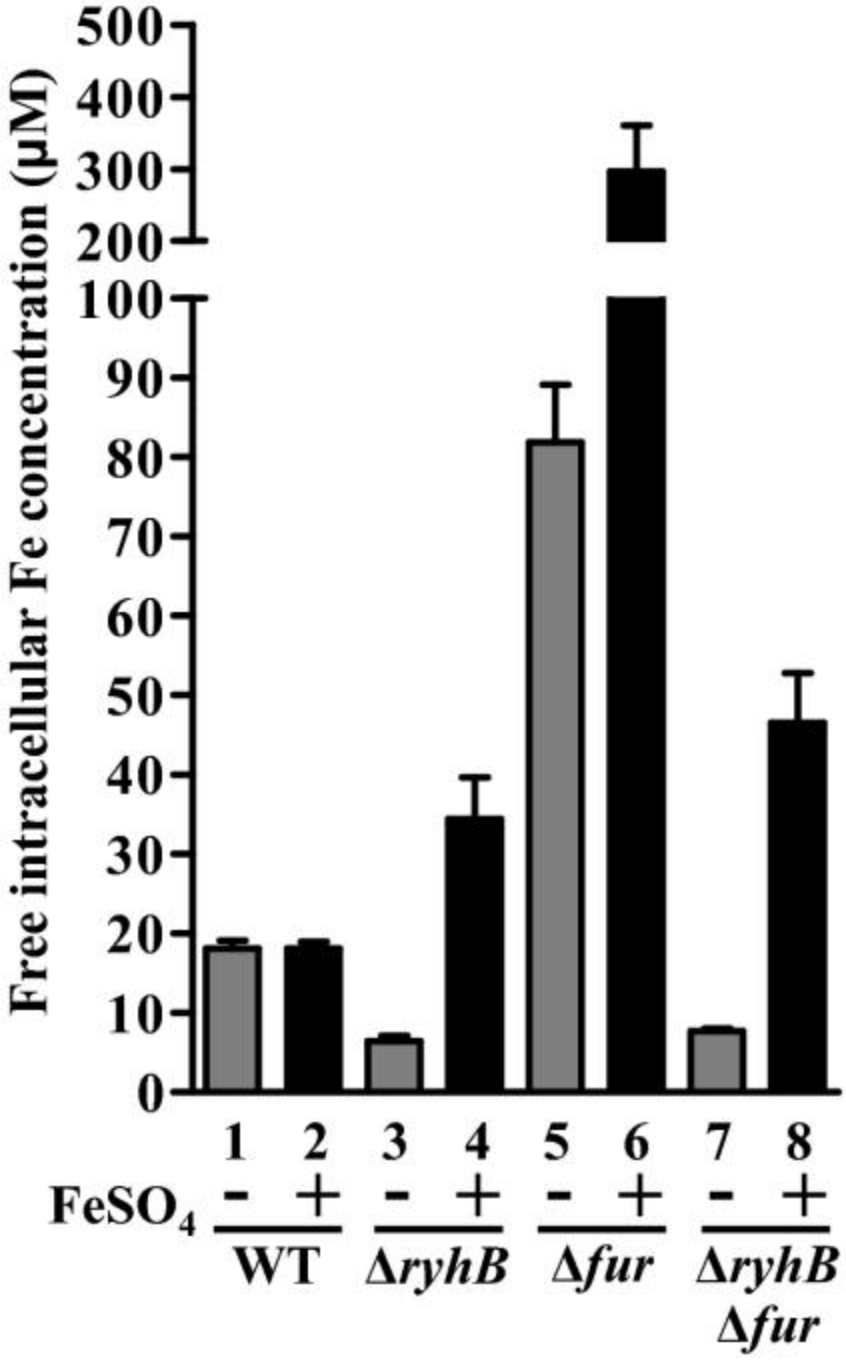

Figure A.2: Concentration of intracellular free iron. Cells were grown in the absence or presence of 1 μM of $FeSO_4$ until an OD_{600} of 0.9, at which point they were assayed for free intracellular Fe by EPR. Strains used were EM1055 (WT), EM1238 (Δ*ryhB*), EM1256(Δ*fur*), and EM1257(Δ*ryhB*, Δ*fur*). This figure has been published in collaboration with Dr. Eric Massé [4].

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APPENDIX B: ARE MONONUCLEAR IRON PROTEINS FROM OTHER ORGANISMS SENSITIVE TO H_2O_2?

B.1 INTRODUCTION

There are a number of lactic acid bacteria that can continue to grow despite their production of high levels of H_2O_2 [1, 3, 4]. It is of interest, both evolutionarily and physiologically, to learn how these organisms can maintain the activity of enzymes, such as the mononuclear iron proteins, in the presence of oxidants that would be damaging to the homologous proteins in *E. coli*. Another evolutionary question regarding mononuclear iron proteins is whether eukaryotes have maintained these proteins as Fe-containing enzymes, or if these organisms have evolved to use different metals. These questions were the focus of studies described in this appendix.

B.2 RESULTS

B.2.1 Are mononuclear iron proteins from the lactic acid bacterium *Lactococcus lactis* sensitive to H_2O_2 in vitro?

In an effort to determine the sensitivity of mononuclear iron proteins from bacteria that produce H_2O_2 during growth, I tried to determine growth conditions that would allow *Lactococcus lactis* to produce H_2O_2 while still synthesizing proteins of interest, such as Rpe and DAHP synthase. In order for DAHP synthase to be expressed, the aromatic amino acids should be absent from the medium. I therefore attempted to grow *L. lactis* on a minimal medium that has been described by others as a means to study this organism's physiology [2]. Despite making the medium as described, the *L. lactis* strain did not well grow on a plate, even after three days at 30°C. When these cells were inoculated into minimal liquid medium, no growth was ever observed. Thus, work

must still be done to determine the composition of a minimal medium that will support the growth of *L. lactis*.

B.2.2 Is Rpe from yeast sensitive to H_2O_2 in vitro?

To test the sensitivity to H_2O_2 of a eukaryotic homolog of a mononuclear iron protein, Baker's yeast was used as a model. Wild-type yeast cells were grown aerobically at 30°C on yeast nitrogen base (YNB), a minimal medium for growing yeast, supplemented with 2% glucose. Cells were harvested aerobically by centrifugation and the pellets were transferred to the anaerobic chamber for extract preparation and assay. Extracts were prepared by bead-beating the resuspended pellets and cleared by centrifugation immediately before being assayed for Rpe activity. When these extracts were treated with 1 mM H_2O_2 for 2 min, Rpe activity was no different than without H_2O_2 treatment (Figure B.1), both having a specific activity of about 0.45 U/mg protein (by comparison, *E.coli* has a specific activity of 4.5 U/mg protein). Addition of a chelator during H_2O_2 treatment had no effect on the activity of yeast Rpe. Thus, it appears that the yeast homolog of Rpe is resistant to H_2O_2 in vitro. The mechanism of this resistance will require further investigation.

B.3 DISCUSSION

While there is little to discuss regarding the lactic acid bacteria, it remains an interesting area of research that should be pursued. However, the preliminary finding that Rpe from Baker's yeast is resistant to H_2O_2 implies that yeast either use an alternative metal in this protein, or that the metal is somehow shielded from H_2O_2. Because the role of the metal in Rpe should be the same in yeast as it is in *E. coli*, solvent exposure of the metal atom should be required in order for it to bind to and activate the substrate. Therefore, it seems unlikely that yeast employs some shielding mechanism, but this idea remains a formal possibility. More work is necessary to determine the mechanism by which yeast Rpe remains active in the presence of H_2O_2.

B.4 EXPERIMENTAL PROCEDURES

B.4.1 Reagents

Amino acids, catalase (from bovine liver), diethylenetriaminepentaacetic acid (DTPA), α-glycerophosphate-triosephosphate isomerase from rabbit muscle, MOPS and associated medium components, β-nicotinamide adenine dinucleotide reduced disodium salt (NADH), D-ribose 5-phosphate disodium salt, D-ribulose 5-phosphate disodium salt, transketolase (from yeast), and 30% H_2O_2 were from Sigma. Glycylglycine was from Acros Organics. Yeast Nitrogen Base, dextrose, and Tris-HCl were from Fisher.

B.4.2 Growth medium

Luria-Bertani (LB) medium contained 10 g tryptone, 10 g NaCl, and 5 g yeast extract per liter. Minimal SA medium was a MOPS medium prepared according to [2] supplemented with 0.2% glucose, 0.5 µg/mL thiamin and 0.5 mM histidine. Other amino acids were added at 0.5 mM where indicated.

B.4.3 Strains and growth

Lactococcus lactis strain IL1403 was a gift from Dr. John Cronan. *L. lactis* was streaked on LB glucose solid medium and incubated overnight at 30°C. Single colonies were picked from the LB plate and onto Minimal SA plates, which were then placed at 30°C.

The Baker's yeast, *Saccharomyces cerevisiae*, wild-type strain X-2180-1a was a gift from Dr. Peter Orlean. The yeast was streaked onto solid Difco YNB medium containing 2% glucose. For Rpe activity determination, an overnight culture of X-2180-1a was inoculated using a single colony into 5 mL liquid YNB + 2% glucose at 30°C. The overnight was then diluted into 15 mL of the same medium to 0.005 OD and grown

to 0.1 OD at 30°C. This pre-culture was then diluted to 0.0015 OD in 50 mL YNB + 0.2% glucose and grown at 30°C to 0.4 OD.

B.4.4 Preparation of yeast cell extracts

When yeast cells reached 0.4 OD as described above, the culture was chilled on ice for 20 minutes and then spun for 8 minutes at 4,000 rpm at 4°C. After removing the supernatant, the cell pellet was resuspended in 50 mL ice-cold 50 mM glycylglycine (pH 8.5) and spun again for 8 minutes at 4°C. The supernatant was discarded and the pellet was moved into an anaerobic chamber. The pellet was then resuspended in 2 mL anaerobic glycylglycine and spun 3 minutes at 14,000 x g at 4°C. The supernatant was again discarded, and the pellet was resuspended in 2 mL glycylglycine containing 1 mM DTPA and the suspension was transferred to a glass tube. Next, 0.5 mm glass beads (acid-washed, rinsed, and thoroughly dried before use) were added to about 1 cm below the level of the liquid and the sample was vortexed for 30 seconds, then placed on ice for 30 seconds. The vortexing and icing combinations was repeated four additional times. The liquid was then poured off, leaving the beads behind, and spun for 3 minutes at 14,000 x g at 4°C. The supernatant was then assayed for Rpe activity immediately.

B.4.5 Rpe assay

Rpe was assayed by diluting the yeast extract 1:10 in 50 mM glycylglycine, then dilution again to a final dilution factor of 1:200 to a reaction mixture containing 50 mM glycylglycine buffer, pH 8.5, 5 mM DTPA, 1 unit of α-glycerophosphate dehydrogenase and 10 units of triosephosphate isomerase (obtained as a pre-mixed suspension), 1 mM ribulose 5-phosphate, 1 mM ribose 5-phosphate, and 0.2 mM NADH in a final reaction volume of 500 µL in a sealed, anaerobic cuvette. The mixture also contained 1 unit of transketolase from a stock of 5 units/mL that was pre-treated with 0.2 mM $MgCl_2$ and 2 mM thiamin pyrophosphate. Absorbance was measured at 340 nm.

B.4.6 Treatment of yeast Rpe with H_2O_2

Undiluted extracts prepared above were diluted 1:5 in buffer containing 1 mM H_2O_2 and incubated at room temperature for 2 minutes. (100 µL extract into 350 µL 50 mM glycylglycine containing 50 µL 10 mM H_2O_2). Aliquots of 90 µL were taken at regular intervals and added to 10 µL of a 1:100 dilution of catalase. Rpe activity was then assayed as described previously.

B.5 FIGURES

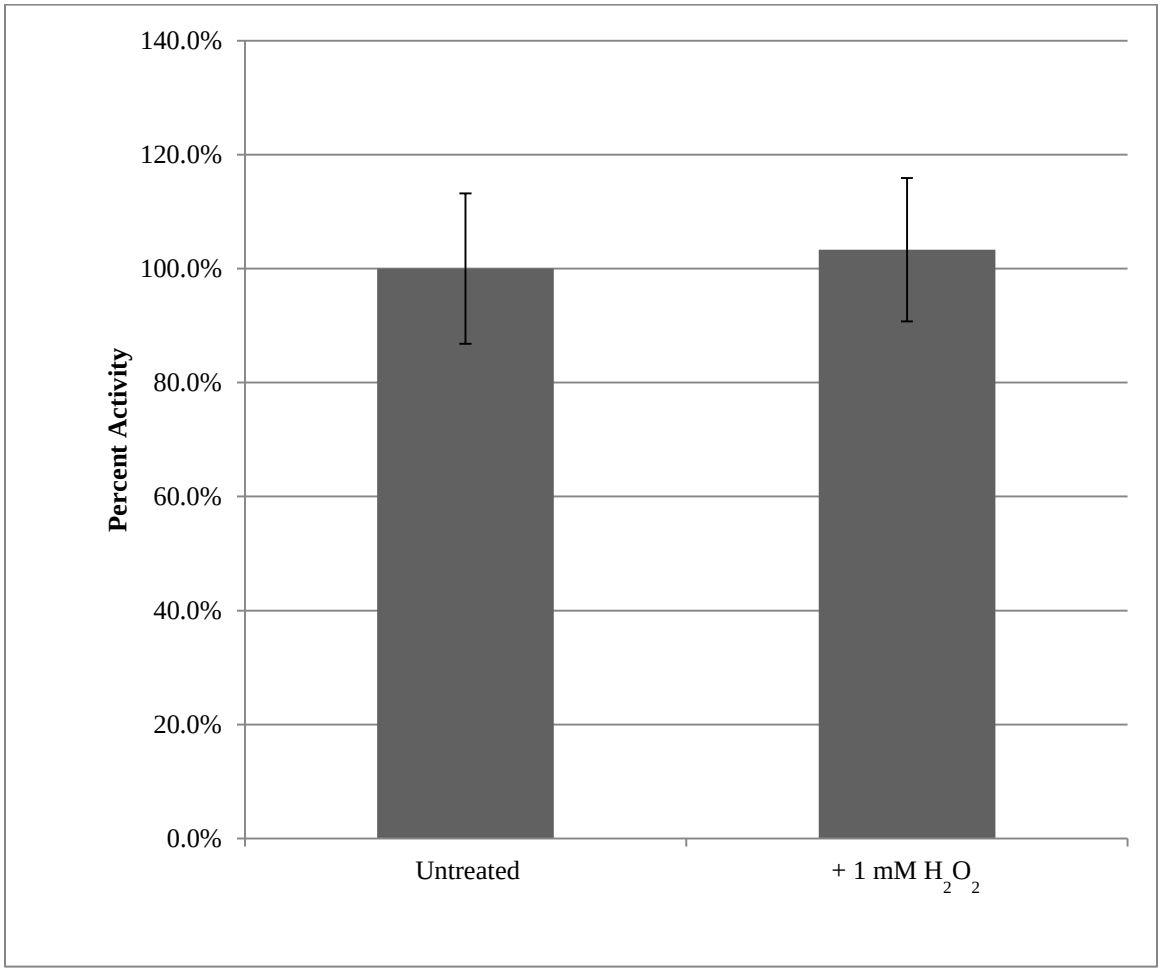

Figure B.1: Rpe from yeast is resistant to H_2O_2. Cultures were grown overnight at 30°C in YNB medium with 2% glucose. Overnight cultures were used to inoculate 15 mL culture in the same medium to 0.01 OD and grown at 30°C to about 0.1 OD. These cultures were then used to inoculate 50 mL of the same medium to 0.0015 OD and cells were grown at 30°C to about 0.4 OD. Cells were then harvested by centrifugation of remaining culture for 8 min at 6,000 x g at 4°C. Pellets were washed with 50 mL ice-cold 50 mM glycylglycine and spun again for 8 min. Pellets were then moved to the anaerobic chamber and assayed for Rpe activity before and after treatment with 1 mM H_2O_2. 1 mM DTPA was added during H_2O_2 treatment to block remetallation. Error bars represent standard deviation from the mean of three independent measurements. Specific activity of untreated sample was 0.448 U/mg protein.

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