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THE MECHANISM BY WHICH MANGANESE PROTECTS *ESCHERICHIA COLI* FROM HYDROGEN PEROXIDE

BY

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DISSERTATION

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ABSTRACT

The goal of this study was to understand the mechanism by which manganese protects cells against reactive oxygen species, particularly H_2O_2. We showed while manganese transport mutants, *mntH*, have no growth defect, *mntH* mutants in strains that cannot scavenge H_2O_2, Hpx$^-$ *mntH*, failed to grow when cultured aerobically. Thus manganese import is only crucial when cells are stressed with H_2O_2; cells appear to use iron to metallate mononuclear enzymes otherwise. Other workers have observed that manganese improves the ability of a variety of microbes to tolerate oxidative stress, and the prevailing hypothesis is that manganese does so by chemically scavenging hydrogen peroxide and/or superoxide .We found that manganese does not protect peroxide-stressed cells by scavenging peroxide. Instead, the beneficial effects of manganese correlate with its ability to metallate mononuclear enzymes. Because iron-loaded enzymes are vulnerable to the Fenton reaction, the substitution of manganese may prevent protein damage. Accordingly, during H_2O_2 stress, mutants that cannot import manganese and/or are unable to sequester iron suffer high rates of protein oxidation.

To directly test our hypothesis, I studied three functionally distinct mononuclear enzymes: peptide deformylase (PDF), threonine dehydrogenase (TDH) and cytosine deaminase (CDA). We showed that these enzymes use iron as their cofactor, and that manganese functionally replaces iron and therefore protects these enzymes when cells are stressed with H_2O_2. We believe iron is frequently overlooked due to quick oxidation of ferrous iron to the insoluble ferric form in aerobic buffers. There are over two hundred mononuclear enzymes in *E. coli*, quite a few of which could be using iron in non-stressed conditions and thus during H_2O_2-stressed conditions could be using manganese. This implies that damage to iron-loaded enzymes is a global problem for cells. As we have demonstrated before, manganese import is critical for cell survival under H_2O_2 stress. In fact, the key metabolic failure of Hpx$^-$ *mntH* cells is due to lack of PDF activity: overexpressing PDF allows these cells to grow. We also demonstrated that these enzymes use manganese as their

cofactor in iron-starved but H_2O_2-scavenging cells, which precludes the role of manganese as a scavenger. This conclusion is also supported by the ability of cobalt to relieve the cellular dependence on manganese during H_2O_2 stress.

In this study, we also demonstrated that apo-PDF and apo-TDH are sensitive to H_2O_2. Both PDF and TDH have a metal-coordinating cysteine residue. While H_2O_2 is known to oxidize free cysteine, it does so at a maximal rate of 2 M^{-1} s^{-1} at neutral pH (half-life of inactivation at 1 μM H_2O_2 ~ 96 h), which is not fast enough to be physiologically relevant. In contrast, we have demonstrated that PDF and TDH are oxidized at a physiologically relevant rate of 1000-1300 M^{-1} s^{-1} (half-life of inactivation at 1 μM H_2O_2 ~ 10 min). These two enzymes are the first examples of proteins with cysteine residues that react with H_2O_2 at such a fast rate. We believe that H_2O_2-senstive active site cysteine residues in these enzymes are preferentially oxidized by H_2O_2 as a mechanism to spare other protein residues from irreversible covalent damage. This oxidized cysteine can then be later repaired by a reductant. Consistent with this idea, we have shown that oxidized inactivated proteins can be easily restored to full activity by a reductant *in vitro*. We have also only been able to retrieve proteins with cysteinyl residues in the over-oxidized state, which implies that the cell repairs oxidized proteins quite rapidly *in vivo*.

To my family and loved ones

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TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION 1

1.1 IRON METABOLISM 1
1.1.1 Importance of iron in biology 1
1.1.2 Iron-dependent chemistry performed by cells 1
1.1.3 Other transition metals can also participate in similar chemistry 3
1.1.4 Importance of iron is often overlooked due to ferrous oxidation in buffers 4
1.1.5 How is *in vivo* metal specificity determined? 5
1.1.6 Trafficking of iron and other metals 7
1.1.7 How do cells cope with iron starvation? 7
1.2 SOURCES OF REACTIVE OXYGEN SPECIES 8
1.2.1 Internal sources of ROS 8
1.2.2 External sources of ROS 10
1.3 IRON-DEPENDENT TOXICITY VIA REACTIVE OXYGEN SPECIES 11
1.3.1 Reaction with O_2^- 11
1.3.2 Reaction with H_2O_2 – the Fenton reaction 11
1.4 TYPES OF ROS DAMAGE 12
1.4.1 DNA damage 12
1.4.2 Protein damage 13
1.5 PROTECTION AGAINST ROS DAMAGE 15
1.5.1 Scavengers 15
1.5.2 OxyR regulon 17
1.5.3 SoxRS regulon 18
1.6 IMPORTANCE OF MANGANESE IN REGARDS TO ROS 19
1.6.1 Dismutation of ROS 19
1.6.2 Iron-independent metabolism 19
1.7 SCOPE OF THIS THESIS 20
1.7.1 Manganese uptake is required when cells are stressed with H_2O_2 20
1.7.2 Manganese protects mononuclear enzymes, during H_2O_2 stress, by displacing iron 21
1.8 TABLES 22
1.9 FIGURES 24
1.10 REFERENCES 26

CHAPTER 2: MANGANESE IMPORT IS A KEY ELEMENT OF THE OxyR RESPONSE TO HYDROGEN PEROXIDE IN *ESCHERICHIA COLI* 41

2.1 INTRODUCTION 41
2.2 RESULTS 42
2.2.1 Manganese import into unstressed cells is minimal and expendable 42
2.2.2 Cells require manganese import during H_2O_2 stress 44
2.2.3 Manganese import is not required to scavenge reactive oxygen species 46

2.2.4 Manganese does not protect DNA from oxidative damage 48
2.2.5 Manganese is required by the cell to protect enzymes 48
2.3 DISCUSSION 50
2.3.1 Are manganese and iron alternative cofactors for mononuclear-metal enzymes? 50
2.3.2 Manganese is not an efficient scavenger of ROS 52
2.3.3 The OxyR regulon is dedicated to the suppression of Fenton chemistry 53
2.4 EXPERIMENTAL PROCEDURES 55
2.4.1 Reagents 55
2.4.2 Bacterial growth 55
2.4.3 Strains and strain construction 56
2.4.4 Enzyme assays 57
2.4.5 Inductively coupled plasma (ICP) measurement on intracellular manganese 58
2.4.6 H_2O_2 concentration measurement 58
2.4.7 Cell viability 59
2.4.8 H_2O_2 killing assay 59
2.4.9 Protein carbonylation 59
2.5 TABLE 61
2.6 FIGURES 63
2.7 REFERENCES 72

CHAPTER 3: APO AND IRON-CHARGED MONONUCLEAR ENZYMES ARE RAPIDLY OXIDIZED BY H_2O_2, AND ARE PROTECTED BY MANGANESE 78

3.1 INTRODUCTION 78
3.2 RESULTS 79
3.2.1 PDF, TDH and CDA are sensitive to H_2O_2 *in vitro* 79
3.2.2 PDF, TDH and CDA are also sensitive to H_2O_2 *in vivo* 80
3.2.3 PDF overexpression rescues Hpx^- *mntH* and Hpx^- *dps* cells 81
3.2.4 Manganese protects mononuclear enzymes by functionally replacing iron in the active site 82
3.2.5 Cobalt supplementation also rescues Hpx^- *mntH* and Hpx^- *dps* cells, and protects against protein carbonylation 84
3.2.6 Apo-PDF and apo-TDH are sensitive to H_2O_2 84
3.2.7 Preferential oxidation of active site cysteine protects further damage to PDF 86
3.2.8 Repeated rounds of damage can irreversibly inactivate PDF and TDH 87
3.3 DISCUSSION 88
3.3.1 Mononuclear enzymes are sensitive to H_2O_2 88
3.3.2 Enzyme active site sulfhydryls are targets of H_2O_2 90
3.3.3 Role of manganese in mononuclear enzymes 92
3.4 EXPERIMENTAL PROCEDURES 94
3.4.1 Reagents 94
3.4.2 Bacterial growth 94

3.4.3 Strains and strain construction 95
3.4.4 Plasmid construction 95
3.4.5 Protein purification 96
3.4.6 Enzyme assays 97
3.4.7 *In vitro* studies....98
3.4.8 *In vivo* H_2O_2 exposure....100
3.4.9 Protein carbonylation....100
3.4.10 Tracking PDF polypeptide *in vivo* 101
3.5 TABLES 102
3.6 FIGURES....104
3.7 REFERENCES 118

CHAPTER 4: CONCLUSIONS 122

4.1 SUMMARY OF CURRENT WORK....122
4.1.1 The role of manganese in protecting cells against reactive oxygen species 122
4.1.2 Mechanism of manganese protection against ROS 123
4.1.3 Enzymes with metal-coordinating cysteinyl residues are prone to rapid oxidation 123
4.2 POSSIBLE FUTURE WORK 124
4.2.1 Which enzyme is responsible for the aromatic amino acid auxotrophy?....124
4.2.2 How does manganese relieve the leucine auxotrophy? 125
4.2.3 Do iron-dependent organisms metallate mononuclear enzymes with manganese?....126
4.2.4 Do eukaryotes continue to metallate mononuclear enzymes with iron? 128
4.2.5 How are cysteinyl-residues re-reduced *in vivo*? 128
4.2.6 Why do cells require the induction of GpmM during H_2O_2 stress?....129
4.2.7 How does manganese continue to be transported in to *mntH* cells?....130
4.3 FIGURES....131
4.4 REFERENCES 137

CURRICULUM VITAE....140

CHAPTER 1: INTRODUCTION

1.1 IRON METABOLISM

1.1.1 Importance of iron in biology

Life evolved on Earth around 3.8 billion years ago, when oxygen was absent from the atmosphere and ferrous iron, along with sulfur, was plentiful [2, 68]. In this anaerobic environment, the reducing power of iron and sulfur allowed the first primitive, chemoautotrophic life to originate [158, 159]. These ancient organisms recruited iron and sulfur into clusters embedded in primitive proteins, such as ferrodoxins (electron transfer) and dehydratases (dehydration reactons) [68], and used these proteins to conduct difficult and biologically important reactions. Over 2.5 billion years ago, oxygen, a by-product of photosynthesis, started to accumulate in the atmosphere [85, 90]. This oxygen at first oxidized ferrous iron in the environment, making iron unavailable for cells. Cells adapted by evolving iron import systems, such as siderophores, to counter the unavailability of ferrous iron, rather than using a different element or process to replace the role of iron. In the present day, with a few exceptions, organisms are dependent on iron for a vast variety of different cellular processes [135]. This requirement for iron can thus be traced back to the origins of life: present-day organisms inherited the requirement for iron to carry out efficient metabolism from ancient organisms.

1.1.2 Iron-dependent chemistry performed by cells

Iron participates in two different classes of reactions inside cells: redox and non-redox based reactions. Versatility of iron, in the different types of function it performs inside the cell, stems from its chemical properties. Iron is a transition element with an unfilled *d*-orbital (six electrons), due to which iron can exist in oxidation states ranging from -2 to +6. The ability to interconvert among different

oxidation states provides iron with tremendous flexibility: 1) iron can participate in various electron transfer reactions inside cells and 2) iron can bind to a wide variety of ligands, six at a time (coordination number of six) [38].

1.1.2.1 Redox based reactions

Iron is known to be used as a prosthetic group in three different classes of enzymes: 1) heme-based enzymes such as catalases, 2) iron-sulfur cluster enzymes such as succinate dehydrogenase, and 3) mononuclear enzymes such as iron-containing superoxide dismutase. Iron-sulfur clusters are one of the oldest complexes still in use in biology. The reduction potential of iron-sulfur clusters can range from -0.6 V to + 0.45 V [19]. Owing to this broad range, iron-sulfur clusters are widely used in biology for electron-transfer reactions e.g. in hydrogenases, nitrogenases and ferredoxins [92].

Catalases are one of the most well known examples of heme-based enzymes, the enzyme first being studied in 1901 [97]. The reaction performed by catalase is a two-step process: 1) Hydrogen peroxide reacts with porphyrin-Fe^{III} to produce water and Compound I ($Por^{\cdot+}$-Fe^{IV}=O), 2) after which another molecule of hydrogen peroxide reacts with Compound I to regenerate porphyrin-Fe^{III} along with water and oxygen [54]. The flexibility afforded by iron, to convert between two oxidation states quickly, makes catalase one of the most efficient enzymes known in biology. Iron performs an equally important redox role in iron-containing superoxide dismutase and ribonucleotide reductase, but in a heme-independent manner. In these enzymes, iron cycles through the ferric and ferrous form during the course of catalysis [27, 140, 151]. It is important to note that these enzymes are only functional with iron as their cofactor, and no other metal can be used as a substitute–the choice and arrangement of ligands coordinating the metal are specific to iron. This is because other redox-active metals may not be poised at the right potential to interact with the substrate/product couple.

1.1.2.2 Non-redox based reactions

Cells are also known to use iron in a redox-independent manner, such as in dehydratases. Dehydratases are iron-sulfur-cluster containing enzymes, where one of the iron atoms is exposed to solvent and bound to a water molecule. This exposed iron binds substrate and is the site at which catalysis takes place [92]. With a coordination number of six, iron has tremendous geometric flexibility in binding ligands. In the case of dehydratases, iron switches from the tetrahedral to the octrahedral (six-coordinate) conformation upon binding the substrate, with negligible use of energy since no bonds are broken (Figure 1.1)[65, 68]. During the course of catalysis the cationic iron also acts as a Lewis acid to help pull away the anionic hydroxyl group, while at the same time a base withdraws a hydrogen atom from the proximal carbon. Overall this results in a net dehydration reaction. Unlike in redox-based reactions, the solvent-exposed iron does not go through a change in oxidation state but instead provides a local positive charge to assist in catalysis, serves as an electron sink, and helps in substrate binding.

Dehydratases are not the only enzymes that use iron in a non-redox manner. Several enzymes have been shown to be able to use iron in a similar way *in vitro*, including isocitrate dehydrogenase [110], methionine aminopeptidase [22], alcohol dehydrogenase [83], and peptide deformylase [9]. The overall role of iron in these enzymes is similar to as in dehydrogenases: to provide a local positive charge, serve as an electron sink, and help in substrate binding (Figure 1.2).

1.1.3 Other transition metals can also participate in similar chemistry

Other first-row transition metals can also partake in reactions similar to iron. This is not surprising since the first-row transition elements share similar characteristics: these metals have a ligand coordination number up to six, and most of these metals have a partially filled *d*-orbital, with a similar size and valence. Multiple metals, such as cobalt, iron, manganese, nickel and zinc, have been shown to activate the same enzymes *in vitro* such as methionine aminopeptidase [22], transketolase

[136, 138] and isocitrate dehydrogenase [110]. In such mononuclear enzymes the metal does not change its redox state but rather helps in substrate binding and provides a local positive charge (Figure 1.2).

Unlike non-redox-based enzymes, redox-based enzymes require the use of their cognate metal. For instance no other metal can replace the role of manganese in manganese-containing superoxide dismutase (SOD) and result in a functional enzyme [145]. However, we see examples in biology where other first-row transition metals are used to perform similar redox-based chemistry in differing isozymes. Cells contain enzymes such as manganese SOD or copper/zinc SOD that are just as efficient at their function as is iron-containing SOD. Even in the case of ribonucleotide reductase, cells have several different isozymes, where NrdEF has recently been shown to be functionally more active with manganese than with iron [13, 101].

1.1.4 Importance of iron is often overlooked due to ferrous oxidation in buffers

Since other metals can perform chemistry similar to that of iron, the requirement for iron in life is not absolute. In fact, organisms such as lactic acid bacteria and *Borrelia burgdorferi* [120] have moved away from using iron and depend on other metals such as manganese. However, in organisms that do depend on iron for viability, the importance of iron in enzymes that require divalent cations is overlooked. Oxidation of ferrous iron happens quite rapidly in aerobic buffers, making it appear as if divalent-cation-utilizing enzymes are not active with iron. Since cellular conditions are reducing in nature, iron exists primarily in the ferrous form inside the cells and is therefore available for binding to divalent-cation-utilizing enzymes. Several enzymes that until recently were thought not to utilize iron have been shown to do so [22, 122]. So, although mononuclear enzymes can use multiple transition metals for activity, it is possible that they predominantly use iron.

1.1.5 How is *in vivo* metal specificity determined?

Cells constantly need to maintain metal homeostasis in order to survive. Transition metals are important cofactors, and cells have evolved sophisticated import and export systems to maintain the concentrations of metals required for growth. Import of too little or too much metal can lead to growth arrest: not enough metal prevents activation of key metalloenzymes, and too much metal can cause toxicity by enzyme mismetallation.

Once imported, metals need to be correctly delivered to their cognate proteins. To date, researchers have made limited progress to determine as to how metal specificity is determined *in vivo*. Since transition metals share similar properties and have been shown to functionally replace one another *in vitro*, it has been challenging to understand how cells are able not only to correctly metallate a metalloenzyme, but to maintain its correct metallation state. Metals in mononuclear enzymes, for instance, have high dissociation rates *in vitro* [136]. How does the cell prevent metal loss from these enzymes, and how does it prevent mismetallation? It seems that several key factors play a role in populating enzymes with the correct metal. These include 1) the availability of the metal inside the cell, 2) the differing affinities of metals for the enzyme active site and 3) the role of metallochaperones and other proteins in delivering and maintaining the correct metal.

Metals are sticky owing to their positive charge and therefore imported metals do not exist in an aqueous state inside the cell but are bound to metabolites and other cell surfaces. In some situations, researchers have observed that if metals are not immediately incorporated into enzymes, any excess metal is often stored. For instance in *E. coli* excess iron is stored by ferritin, a metal storage protein [143].

Metal concentrations inside cells can differ based on different cellular compartments, such as the periplasm and cytoplasm, potentially allowing enzymes to receive the correct metal based on the location these enzymes fold inside cells. Such an example has been seen in cyanobacteria where similar proteins are metallated with either copper or manganese based on if the protein folds in the cytoplasm

(manganese-rich, copper-poor) or in the periplasm (copper-rich, manganese-poor) [144]. It is important to note that this kind of compartmentalization would also prevent a metal from being incorrectly metallated, since only the correct metal would be present in a sufficiently high enough concentration.

Although transition metals share similar chemical properties and can replace one another *in vitro* in mononuclear enzymes, their ligand binding affinities are broadly different. The Irving-William series predicts the stability of metal ligand binding in the following order: Mg^{2+} and Ca^{2+} (weakest binding) $< Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$ [76]. According to this series, copper has the highest ligand binding. In *E. coli*, copper has been shown to interfere with iron binding in iron-sulfur clusters and therefore to inactivate iron-sulfur cluster containing proteins [99]. Cells like *E. coli* have also been reported to have extremely low concentration of copper in the cytoplasm, which makes sense considering copper has the highest affinity for protein ligands, and thus the cell limits exposure of proteins to copper. Cells also carefully control the amount of nickel imported into the cell, owing to the high binding ability of nickel [123].

So far only a few metallochaperones have been found to exist inside cells, including the well-studied copper chaperone CCS [63] and the nickel chaperone HypA [164]. In both of these cases the chaperone is required to deliver the relevant metal *in vivo*. Since both copper and nickel have high ligand-binding affinities, without the presence of chaperones these metals would adhere to adventitious ligands and not be correctly routed to their target proteins, especially considering the low concentrations of both copper and nickel inside cells [100, 114]. In addition to chaperones, cells use other types of proteins to maintain the correct metallation state of enzymes. Recently it has been shown that in *E. coli* YfaE is required for continued ribonucleotide reductase (NrdAB) function when cells are stressed with hydrogen peroxide. Although the YfaE mechanism of action is not known and is being investigated, it seems likely that YfaE can discriminate between iron and manganese and keep NrdAB correctly metallated with iron [62, 101].

1.1.6 Trafficking of iron and other metals

Not much is known about metal trafficking inside cells. While some progress has been made in understanding how copper and nickel are trafficked *in vivo* [28, 123], not much is known about how cells deal with intracellular iron and other transition metals.

Once imported into *E. coli*, iron can be stored by bacteroferritin, ferritin or Dps protein [30, 50]. Although the mechanism by which iron is stored is known, i.e. converted from ferrous to ferric form (Fe_2O_3) [66, 166], the mechanism by which iron is delivered and retrieved from these storage proteins is not understood. Researchers have speculated that proteins such as YggX, CyaY (frataxin homolog) and IscA could possibly be either iron carriers, or involved in iron trafficking. However, till now the roles of these proteins have not been definitely demonstrated.

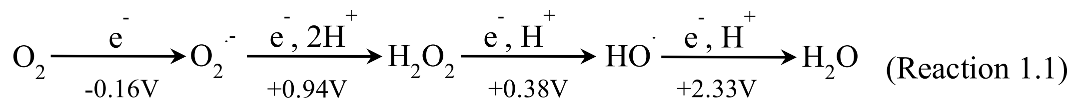
1.1.7 How do cells cope with iron starvation?

Ferric uptake regulator (Fur) is responsible for maintaining iron homeostasis inside *E. coli* and other organisms. Fur protein, bound to iron (II), acts primarily as a transcriptional repressor and limits the amount of iron imported when cells are replete with the metal. Fur does this by repressing TonB transcription: TonB powers high affinity iron-uptake systems [153, 161]. Metallated Fur also represses the transcription of a small ribosomal RNA (RyhB) [104, 155]. RyhB targets the RNA of dispensable iron-containing proteins for degradation in iron-scarce conditions. These proteins include iron-containing superoxide dismutase, aconitase A and fumarase A [103-105, 135, 156]. Some of these proteins have non-iron-requiring isozymes, such as manganese-containing superoxide dismutase and fumarase C, which are upregulated in iron-limiting conditions. RyhB also promotes the synthesis of siderophores [126]. Therefore in iron-limiting conditions, Fur exists primarily in the apo-state, which results in increased RyhB expression and in turn RyhB attempts to spare iron for essential iron-requiring enzymes (e.g. isopropylmalate isomerase, dihydroxyacid dehydratase [104].

Metallated-Fur also represses the expression of *mntH*, the sole dedicated manganese transporter in *E. coli* [117], along with manganese-containing superoxide dismutase (SodA) [57, 152] and manganese-requiring ribonucleotide reductase (NrdEF) [101]. Thus in iron-poor conditions, the cell attempts to shift away from an iron-based metabolism to one that depends on manganese. Although *E. coli* still requires iron for growth, researchers have shown that in extremely low iron conditions, the induction of manganese is critical for survival [51, 101]. These observations raise the question as to whether or not manganese can replace iron in mononuclear metal enzymes.

1.2 SOURCES OF REACTIVE OXYGEN SPECIES

As a consequence of living in an oxygen atmosphere, aerobic and anaerobic organisms are constantly exposed to reactive oxygen species (ROS), such as H_2O_2 and O_2^-, from both internal and external sources. While oxygen by itself is a weak univalent oxidant (-0.16 V O_2/O_2^-), the partially reduced forms of oxygen are much stronger univalent oxidants (Reaction 1.1). These partially reduced species [111] have a sufficiently strong reduction potential to strip electrons from some biomolecules and to cause cellular damage in the process.



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

1.2.1 Internal sources of ROS

Cells that are devoid of ROS scavengers have been shown to have a variety of growth defects when grown in the presence of oxygen [20, 78]. These growth defects show that aerobically grown cells constantly generate ROS and can be poisoned by them. Endogenous reactive oxygen species (ROS) are produced when oxygen adventitiously steals an electron from inside the cell. Initially researchers believed that reactive oxygen species, such as H_2O_2 and O_2^-, were produced as an inadvertent

consequence of partial oxygen reduction by cytochrome oxidase. This hypothesis was later shown not be supported by experimental evidence [109]. However, researchers were able to show that H_2O_2 and O_2^- could be produced when mitochondrial or bacterial membrane vesicles respired *in vitro*, indicating that perhaps the respiratory chain flavoenzymes are responsible for ROS production [23, 72, 107]. This idea is reasonable considering oxygen is a weak univalent oxidant and only a few biomolecules can spontaneously reduce it, such as the univalent redox moieties found in the respiratory chain. Enzymes such as NADH dehydrogenase II and succinate dehydrogenase are able to produce ROS at a substantial rate *in vitro* [108]. However, cells lacking NADH dehydrogenase did not substantially lessen cellular ROS production whereas over-production of NADH dehydrogenase II led to only a modest increase of *in vivo* H_2O_2 formation [131]. Thus the respiratory chain enzymes are not major sources of ROS *in vivo*.

Besides the respiratory chain, flavoenzymes are found in only a handful of other pathways that have sufficient flux to account for endogenous H_2O_2 production. However, it was recently shown that most flavoproteins do not significantly contribute to H_2O_2 formation. Surprisingly, NadB, a flavin-dependent dehydrogenase that is involved in nicotinamide biosynthesis, accounts for 25-30% of endogenous H_2O_2 production [87]. In anaerobic cells NadB uses fumarate as an electron acceptor whereas in aerobic cells it uses oxygen to adventitiously act as an electron acceptor instead, which results in the generation of H_2O_2. Similarly, fumarate reductase, which uses a flavin subunit homologous to NadB, can also produce substantial H_2O_2 in the presence of oxygen [87]. It seems that cells tolerate NadB H_2O_2 production owing to the small flux that goes through this pathway. So why is it that other flavin dehydrogenases, with significantly greater flux, do not produce substantial H_2O_2 *in vivo*? Other significant flavoenzymes are membrane-bound enzymes that donate electrons directly to the quinone pool, unlike NadB. These electrons are then transferred to oxygen through cytochrome oxidases. This arrangement greatly shortens the residence time of electrons on flavins in the respiratory chain as compared to NadB, preventing ROS production.

Besides NadB, menaquinone autoxidation accounts for another 5-10% of H_2O_2 production *in vivo* [86]. Together with NadB, this accounts for only one-third of the H_2O_2 produced inside cells. The source of the remaining two-thirds of H_2O_2 production is not yet known.

1.2.2 External sources of ROS

Organisms are exposed to reactive oxygen species (ROS) in a variety of ways in their environment. Exposure to UV-radiation can lead to the production of ROS in aerobically growing cells [165]. Growth media such as Luria-Bertani broth contain riboflavin, which can generate ROS when exposed to light, in the presence of oxygen [15]. Chemicals, such as ascorbic acid, can also produce ROS in the presence of oxygen: ascorbic acid is frequently used to generate ROS in a lab environment.

Certain organisms produce reactive oxygen species (ROS) as a biological weapon to gain a competitive advantage over other organisms. Organisms such as lactic acid bacteria have been shown to produce millimolar levels of H_2O_2 in their environment by using H_2O_2 producing oxidases such as pyruvate oxidase, lactate oxidase and NADH oxidase [49, 134, 137]. Other organisms produce redox cycling drugs that can also lead to the production of ROS *in vivo*. These drugs can easily enter the cytoplasm of cells and generate ROS by transferring electrons, stolen from flavins or metal centers of redox enzymes, to oxygen [56]. Examples of such redox cycling drugs include plumbagin [157] and juglon [75] excreted by plants, and phenazines excreted by bacteria including *Pseudomonas, Streptomyces* and *Pantoea agglomerans* [150].

Organisms are also believed to encounter ROS in macrophages as part of the oxidative burst. While invading organisms have been shown to require superoxide scavenging enzymes in the periplasm to survive in their hosts [88, 89], the relevance of H_2O_2 production is unclear [8, 142].

1.3 IRON-DEPENDENT TOXICITY VIA REACTIVE OXYGEN SPECIES

Both H_2O_2 and O_2^- are relatively strong univalent oxidants, as compared to oxygen, but these two ROS species by themselves cannot effectively damage organic biomolecules directly. Their *in vivo* toxicity depends on their reaction with iron.

1.3.1 Reaction with O_2^-

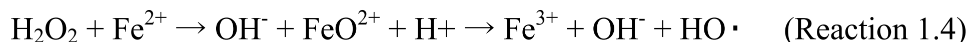
With a reduction potential of +0.94V, thermodynamics indicate that O_2^- might be able to damage a range of biomolecules *in vivo*. However, electrostatic repulsion inhibits the anionic O_2^- from abstracting electrons from electron-rich molecules [12, 37, 69, 128]. Flint and coworkers [39, 40] demonstrated that O_2^- can damage solvent-exposed iron-sulfur containing enzymes by directly reacting with iron itself. These enzymes, with solvent-exposed iron-sulfur clusters, are particularly vulnerable to O_2^- because superoxide's anionic charge is electrostatically attracted to the positive charge on the ironatoms. Thus O_2^- is univalently able to oxidize the cluster and degrade the cluster in the process. O_2^- has been shown to do this at an extremely high second-order rate of 10^6-10^7 [41]. The overall reaction results in H_2O_2 and free ferrous iron, which further causes problems for the cell.

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

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

1.3.2 Reaction with H_2O_2 – the Fenton reaction

The main toxicity of hydrogen peroxide toxicity stems from the Fenton reaction, in which H_2O_2 reacts with ferrous iron, resulting in the formation of hydroxyl radical (Reaction 1.4).



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

With a reduction potential of +2.38V and neutral charge, hydroxyl radical can damage any biomolecule inside the cell – at nearly diffusion-limited rates. The reduction of H_2O_2 is critical for the formation of the hydroxyl radical. In order for H_2O_2 to accept electrons, its dioxygen bond must be broken, which imposes a significant barrier to the reactivity of H_2O_2. However the interaction of hydrogen peroxide with the *d* orbital of iron, and with other transition metals (e.g. copper), can significantly weaken this bond. Catalases work on the same principle in dismuting H_2O_2 [69].

The published rate constant for the Fenton reaction is 76 $M^{-1} s^{-1}$, at pH 3 [160]. Cells have approximately 20 μM ferrous "free" iron that can participate in the Fenton reaction [84, 139, 163], while the steady-state concentration of H_2O_2 is estimated to be around 20 nM *in vivo* [132]. These values suggest that with a constant of only 76 $M^{-1} s^{-1}$, the Fenton reaction is an insignificant oxidative stress factor *in vivo*. However, the Fenton reaction rate constant is severely modulated depending on the ligands ferrous iron is bound to and pH. For instance rate constants of 2000-6000 $M^{-1} s^{-1}$ have been measured at neutral pH, when ferrous iron is bound to DNA [115, 125]. In aerobic habitats, organisms could be exposed to concentrations of H_2O_2 higher than endogenous levels [132]. Thus the damage to cells caused by the Fenton reaction is an extremely important form of oxidative stress.

1.4 TYPES OF ROS DAMAGE

1.4.1 DNA damage

Researchers have reported that growing *E. coli* cells exposed to H_2O_2 *in vivo* show DNA damage, and that DNA-repair mutants are killed quickly [21, 32, 73]. This type of damage is mediated via the Fenton reaction, since cell-permeable iron chelators are able to prevent DNA damage in the presence of H_2O_2 [71]. These compounds chelate iron from DNA and thereby prevent H_2O_2 from generating hydroxyl radicals on the DNA surface [121]. If produced, the hydroxyl radical would

lead to the formation of a variety of adducts such as 8-hydroxyguanine [18] and could also lead to strand cleavage [34, 64]. Therefore hydroxyl-mediated DNA damage could easily lead to cell death.

Since the rate constant for the Fenton reaction is quite high when iron is bound to DNA, over time cells can accumulate substantial DNA damage even at an intracellular concentration of 20 nM intracellular H_2O_2. Indeed, DNA damage proportionally rises with conditions that elevate the levels of intracellular iron, consistent with the Fenton equation. While O_2^- does not damage DNA directly, it can contribute to DNA damage by oxidizing solvent-exposed iron sulfur clusters, increasing the levels of unincorporated iron inside cells [84, 146]. Together, both O_2^- and H_2O_2 can lead to accumulation of substantial amounts of DNA damage via the Fenton reaction, ultimately leading to cell death.

1.4.2 Protein damage

1.4.2.1 Fe-S clusters

In 1986 Carlioz and Touati showed that cells that lack superoxide dismutase activity, and thus accumulate O_2^- *in vivo*, are unable to grow without supplements of branched-chain, aromatic and sulfur-containing amino acids [20]. Cells that cannot scavenge H_2O_2 also require aromatic supplements, and branched-chain supplements in the presence of 8 μM H_2O_2 (J. M. Sobota and J. A. Imlay, unpublished results) [78]. Since only a few processes are affected, the damage caused by these ROS must be specific in nature.

Flint and coworkers were able to demonstrate that solvent-exposed iron-sulfur clusters are particularly vulnerable to O_2^-, damage to which could explain the branched-chain amino acid auxotrophy caused by O_2^- [14, 39, 40]. H_2O_2 also inactivates the branched-chain amino acid pathway by damaging solvent-exposed iron-sulfur cluster containing enzymes, in a manner analogous to the Fenton reaction

[78]. While O_2^- reacts with FeS clusters at a high rate (10^6-10^7 M^{-1} s^{-1}), the rate of inactivation by H_2O_2 is comparatively slow (10^3-10^4 M^{-1} s^{-1}) [41, 78].

Both O_2^- [44-47, 95, 154] and H_2O_2 [78] have been shown to inactivate other solvent-exposed iron-sulfur clusters as well, such as aconitase and isopropylmalate isomerase, while enzymes with buried iron-sulfur clusters are not susceptible to ROS damage. This is presumably because O_2^- and H_2O_2 cannot access these buried FeS clusters. This idea is supported by the observation that substrate is able to protect solvent-exposed iron-sulfur clusters from ROS damage, by preventing O_2^- and H_2O_2 from coming into contact with the cluster [78].

1.4.2.2 Damage to other proteins and polypeptides

Both O_2^- and H_2O_2 have not been shown to damage proteins by direct oxidation at rates that would be physiologically relevant. While H_2O_2 can directly oxidize typical cysteine and cysteinyl-residues, it does so at an extremely slow rate of about 2 M^{-1} s^{-1} at neutral pH [162]. With an intracellular concentration of H_2O_2 estimated at 20 nM, it would take days before H_2O_2 directly oxidizes cysteinyl-residues. However, precedence for a high oxidation rate exists: OxyR, AhpC and OhrR contains thiols that react with H_2O_2 with rates over 10^5 M^{-1} s^{-1} [7, 118]. In these examples it seems that vicinal ligands to cysteine and the surrounding environments of cysteinyl-residues contribute to the high oxidation rate. Therefore it might be possible that some cysteine containing proteins are vulnerable to direct H_2O_2 damage, at rates that are physiologically important. However, to date, no such examples have been show to exist.

ROS species can still lead to physiologically important levels of protein damage by generating hydroxyl radicals via the Fenton reaction. Hydroxyl radicals can oxidize proteins at near diffusion-limited rates of 10^8 to 10^{10} M^{-1} s^{-1} [31]. Metal-catalyzed oxidation, via the hydroxyl radical, has been shown to inactivate alcohol dehydrogenase [141] and iron-containing superoxide dismutase [11] *in vitro*. Hydroxyl radicals can also lead to formation of protein carbonyls, which have been

shown to be present in aerobic bacteria, and are elevated when cells are exposed to H_2O_2 and redox-cycling drugs [36]. However, the significance of protein carbonylation *in vivo* is not yet known.

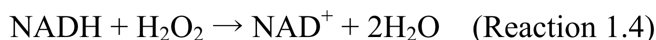
1.5 PROTECTION AGAINST ROS DAMAGE

1.5.1 Scavengers

1.5.1.1 H_2O_2 scavengers

E. coli expresses two catalases, encoded by *katE* and *katG*, as well as an NADH peroxidase, *ahpCF*. All three enzymes are able to scavenge H_2O_2 efficiently. From observations in cells lacking superoxide dismutase activity (*sodA sodB* mutants), it was hypothesized that cells lacking H_2O_2 scavenging ability would also be less oxygen-tolerant. At the time, only catalases (*katE katG* in *E. coli*) were thought to be physiologically relevant scavengers of H_2O_2. However, *katE katG* mutants were shown to be equally viable as wild-type cells and exhibited no growth defects [98, 129]. Therefore it was concluded that endogenous levels of H_2O_2 are not generated to toxic levels inside cells. Later on with the discovery of AhpCF as a potent scavenger of H_2O_2, cells lacking *ahpCF, katE and katG* were shown to be oxygen-sensitive [130]. Thus toxic levels of H_2O_2 are indeed produced endogenously in aerobically growing cells, requiring cells to express H_2O_2 scavenging enzymes.

AhpCF is a two-component NADH peroxidase, with a peroxidatic component, AhpC and an NADH-reducible flavin component, AhpF. H_2O_2 oxidizes a cysteine residue, Cys46, on AhpC, which leads to the formation of a disulfide bond with another cysteine residue, Cys165. This disulfide bond is then transferred to other sulfhydryl residues on AhpC, which AhpF ultimately reduces (Reaction 1.4).



$$NADH + H_2O_2 \rightarrow NAD^+ + 2H_2O \quad \text{(Reaction 1.4)}$$

AhpCF has been shown to be unable to scavenge H_2O_2 at concentrations above 20 μM [132]. This is thought to be because above this concentration of H_2O_2, AhpC oxidation happens at a much faster rate than its reduction by AhpF. Presumably this arrangement is beneficial for the cells in order to prevent exhaustion of cellular NADH levels. Above concentrations of 20 μM H_2O_2, catalases become the primary scavenger of H_2O_2.

So why does the cell rely on AhpCF at lower concentrations of H_2O_2, instead of catalase, especially considering AhpCF requires reducing energy, in the form of NADH, to function? AhpCF has at least an order higher catalytic efficiency of 4×10^7 M^{-1} s^{-1} [116], as compared to 9×10^5 M^{-1} s^{-1} for HPI [59] and 1.3×10^6 M^{-1} s^{-1} for HPII [113]. Therefore the cells would need at least ten-fold more catalase molecules to provide cells with equivalent scavenging ability to AhpCF.

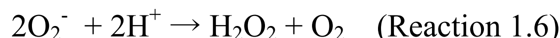
As mentioned earlier, at high H_2O_2 levels, catalases take over the primary responsibility to scavenge H_2O_2 from AhpCF. This makes sense considering catalases have a high K_M for H_2O_2 – in the millimolar range [59]. Catalases dismute H_2O_2 and therefore require no reducing equivalents from the cells (Reaction 1.5).

$$H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2 \quad \text{(Reaction 1.5)}$$

In *E. coli* these three scavengers together maintain *in vivo* H_2O_2 levels to 20 nM [132]. In strains that are devoid of these three H_2O_2 scavengers, H_2O_2 rapidly accumulates to toxic steady-state levels of 0.5-1 μM, at a rate of 14 μM/s [132]. Thus AhpCF, KatE and KatG are enzymes necessary for cells to combat H_2O_2 stress.

1.5.1.2 Superoxide dismutases

While O_2^- can spontaneously dismute to H_2O_2 at a rate constant of 10^5 M^{-1} s^{-1} [43], superoxide dismutases (SOD) can dismute O_2^- at diffusion-limited rates of 2×10^9 M^{-1} s^{-1} [16, 17] (Reaction 1.6).



$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{(Reaction 1.6)}$$

Due to the high reactivity of O_2^-, it is extremely important for the cell to keep O_2^- as low as possible. Most organisms, both aerobic and anaerobic, contain either SODs [106] or superoxide reductases [70].

E. coli contains three SODs: two cytoplasmic SODs, manganese-containing superoxide dismutase and iron-containing superoxide dismutase, and one periplasmic SOD, copper/zinc-containing superoxide dismutase. MnSOD and FeSOD are responsible for dismuting cytosolic O_2^-. Both of these enzymes are structurally and kinetically similar and are only active with their cognate metals. FeSOD is expressed anaerobically and is responsible for dismuting O_2^- if cells are suddenly exposed to oxygen. FeSOD is negatively regulated by RyhB [104], so in iron-replete conditions metallated Fur keeps FeSOD transcription on. MnSOD, on the other hand, is negatively regulated by Fur (as well as ArcA and FNR [26, 57]) so in iron-limited conditions and/or in an aerobic environment MnSOD is the primary SOD for the cell [26, 35, 112]. Such regulation ensures that the cell has superoxide dismutase activity under a wide variety of conditions, which underlines the importance of these enzymes for cellular metabolism.

1.5.2 OxyR regulon

The transcription factor OxyR is the primary global H_2O_2 sensor in *E. coli*. OxyR is activated by H_2O_2 at an extremely high rate of 10^7 M^{-1} s^{-1}, where oxidation of OxyR sulfhydryl residue leads to the formation of a disulfide bond [25, 93, 167]. This activated form of OxyR induces the expression of at least 20 genes (Table 1.1). These genes include scavenging enzymes, such as AhpCF and KatG, and enzymes involved in iron homeostasis, such as Fur and Dps. Dps (DNA-binding protein from starved cells) is an intracellular iron chelator that limits the amount of free iron in the cytoplasm [166]. Dps, together with H_2O_2 scavengers, YaaA and Fur, limit the amount of Fenton-mediated damage inside the cell [96, 115].

OxyR also induces the expression of *mntH*, the sole dedicated manganese importer inside cells [81]. Manganese supplementation has been shown to be

beneficial for H_2O_2-stressed cells. Researchers conjectured that this protective ability of manganese is presumably by manganese scavenging H_2O_2 [79, 133, 148]. Other genes that OxyR induces include the Suf system, an iron-sulfur cluster assembly system critical for cell survival under H_2O_2 stress because H_2O_2 poisons the Isc system [77, 168], along with glutaredoxin, thioredoxin and a variety of genes of unknown function. Although the function of proteins these genes encode is unknown, expression of these genes is thought to be important during H_2O_2 stress [96].

1.5.3 SoxRS regulon

SoxRS, a two component regulatory system, was originally believed to be a superoxide sensor. Since SoxR is activated by the oxidation of its iron-sulfur cluster [53, 58, 147], and at the time it was well known that O_2^- could quickly damage FeS clusters [41, 45, 46, 91], it was thought possible that cells would use SoxRS to detect the presence of O_2^-. SOD mutants, which accumulate toxic levels of O_2^-, were later shown to activate the SoxRS response [48, 94]. Furthermore, SoxRS was shown to induce the expression of MnSOD and other O_2^- resistant isozymes [53, 102, 119, 147], which further suggested the role of SoxRS as an O_2^- sensor.

However, since then researchers have shown that redox cycling drugs can anaerobically turn on the SoxRS response [33, 55], and that these drugs activate SoxRS at a much higher rate – forty fold by paraquat as compared to only three fold in SOD mutants [48, 55]. This suggests that redox-cycling drugs are the primary activators of the SoxRS response, rather than O_2^- alone. Since redox-cycling drugs can lead to the production of O_2^-, it makes sense for the cellular response against redox-cycling drugs to induce the expression of genes, like MnSOD, to combat O_2^- as well. However, SoxRS also turns on a range of other genes, such as drug efflux pumps (Table 1.2) [102, 119], that are not important in dealing with O_2^- but rather with redox cycling drugs. Thus, the SoxRS regulatory system provides bacteria with an effective response against the toxic effects of redox-cycling drugs.

1.6 IMPORTANCE OF MANGANESE IN REGARDS TO ROS

1.6.1 Dismutation of ROS

Manganese has been shown to have a beneficial effect against ROS in a wide variety of organisms. In *Salmonella typhimurium, Streptococcus pneumoniae* and *Neisseria gonorrhoeae*, manganese supplementation and import can help cells tolerate the effects of high doses of H_2O_2 [79, 133, 148]. Manganese supplementation can also protect cells against O_2^- stress [1, 24, 60] and protect against damaging effects of gamma radiation [29]. Also, OxyR has been shown to control manganese uptake in both *E. coli* and *Salmonella*, suggesting that manganese import is important during H_2O_2 stress [81].

The mechanism by which manganese protects these organisms against ROS *in vivo* is not yet known. However, manganese has been shown to scavenge both H_2O_2 and O_2^- *in vitro* [10, 52, 61]. Therefore, researchers have speculated that perhaps cells import manganese to provide cells with additional ROS scavenging ability [1, 5, 24, 29, 60, 61, 74, 80, 124, 127, 133, 149]. While the rate of H_2O_2 dismutation by manganese *in vitro* can be quite high in ideal situations [10] and does not lead to the production of hydroxyl radical, it is unlikely that cells use a metal to scavenge ROS when cells express dedicated enzymes for the same purpose. Therefore, the aim of this thesis is to evaluate the protective role of manganese *in vivo*.

1.6.2 Iron-independent metabolism

Organisms, such as *Lactobacillus plantarum*, have been shown to import millimolar amounts of manganese [4]. These organisms import little to no iron [3], and not only can tolerate ROS but produce and release high amounts of H_2O_2 into their environment – to poison their competitors [6]. Other organisms, such as *Borrelia burgdorferi*, depend on a manganese-dependent – and thus iron-independent – metabolism presumably as a survival mechanism against the immune system of its hosts [42, 120].

The dependence of most of present day life on iron is likely an evolutionary requirement that can be traced to the beginning of life, which began in an anaerobic world where iron was readily available. Perhaps *Lactococcus* and *Borrelia burgdorferi* are examples of evolution moving life away from dependence on iron, a metal whose availability is limited and contributes to the formation of toxic species in the presence of oxygen. This transition towards an iron-independent metabolism is more apparent in organisms that can use both iron and manganese. In *E. coli* manganese uptake is regulated by the Fur regulon [81, 117], indicating that cells find it necessary to import manganese in iron-limited conditions. This regulation suggests that some of the iron-dependent enzymes in *E. coli*, and other organisms, can use manganese as a substitute for iron. *E. coli* also has mangano-isozymes for iron-requiring redox enzymes, such as NrdEF and MnSOD. However, *E. coli* still requires some iron for growth and cannot solely depend on manganese alone. Perhaps in the future organisms like *E. coli* will also rely solely on an iron-independent metabolism.

1.7 SCOPE OF THIS THESIS

1.7.1 Manganese uptake is required when cells are stressed with H_2O_2

Manganese has been shown to have a protective effect against H_2O_2 and O_2^- in a variety of organisms. Since manganese can dismute both H_2O_2 and O_2^- *in vitro*, manganese is believed to protect cells by dismuting ROS *in vivo* as well. Furthermore, OxyR regulates manganese uptake in cells, suggesting that cells find it important to import manganese during ROS stress. Since cells express dedicated ROS scavengers, it seems unlikely that manganese would be imported simply to scavenge ROS *in vivo*. In this work we decided to test if manganese import is essential during H_2O_2 stress and if so, why? This work disproved the role of manganese as a ROS scavenger *in vivo* and showed that manganese import was required during H_2O_2 stress to protect proteins – presumably by displacing iron in mononuclear enzymes and thus preventing Fenton-mediated damage to enzymes.

1.7.2 Manganese protects mononuclear enzymes, during H_2O_2 stress, by displacing iron

The goal of this part of the work was to directly show, with examples, that iron is the cofactor of choice for mononuclear enzymes in the absence of ROS stress, and that manganese import is required during H_2O_2 stress to protect these enzymes by displacing iron. I was able to demonstrate that peptide deformylase, threonine dehydrogenase and cytosine deaminase are three such examples of enzymes. Together with ribulose phosphate epimerase [136], we now know four enzymes that are sensitive to H_2O_2 stress when iron is used as a cofactor and are protected when manganese is used in place of iron for activity.

For peptide deformylase and threonine dehydrogenase, I was also able to show that the active site cysteine in these enzymes is extremely sensitive to oxidation by H_2O_2, and that the manganese-bound enzyme – unlike the iron-bound enzyme – is resistant to this type of damage.

1.8 TABLES

Table 1.1 Selected members of the OxyR regulon [67]

Function	Enzyme
H_2O_2 scavengers	AhpCF KatG
Heme synthesis	Ferrochetalase
Iron chelators	Dps
Iron regulation	Fur
Iron homeostasis (unknown function)	YaaA
Manganese importer	MntH
Disulfide reduction	Thioredoxin C Glutaredoxin A Glutathione reductase

Table 1.2 Selected members of the SoxRS regulon [67]

Function	Gene
Dehydratase isozymes resistant to ROS	*fumC* (Fumarase C) *acnA* (Aconitase A)
Drug efflux and/or resistance	*acrAB* (drug efflux pump) *tolC* (OMP component of drug efflux pump) *micF* (OmpF antisense sRNA) *marAB* (multiple antibiotic resistance operon) *nfnB* (nitroredutase) *rimK* (modification of ribosomal protein S6)
Superoxide dismutase	*sodA*
Iron regulation	*fur*

1.9 FIGURES

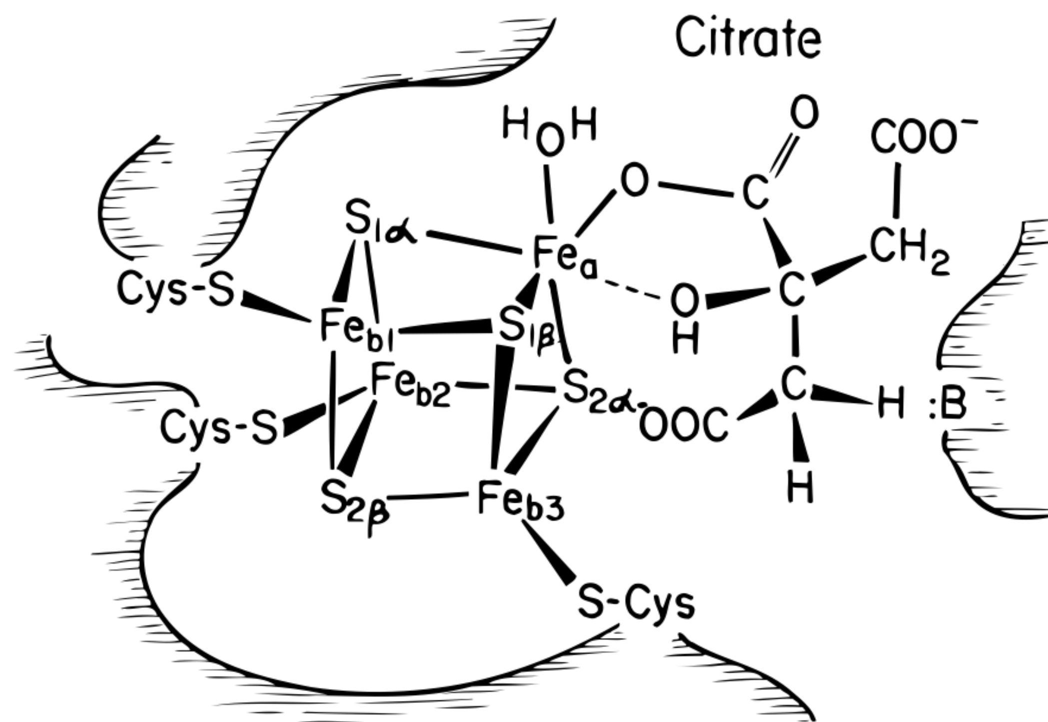


Figure 1.1. Six-coordinate ligand conformation of the solvent-exposed iron, Fe_a, to citrate in the [4Fe-4S] cluster of aconitase [82].

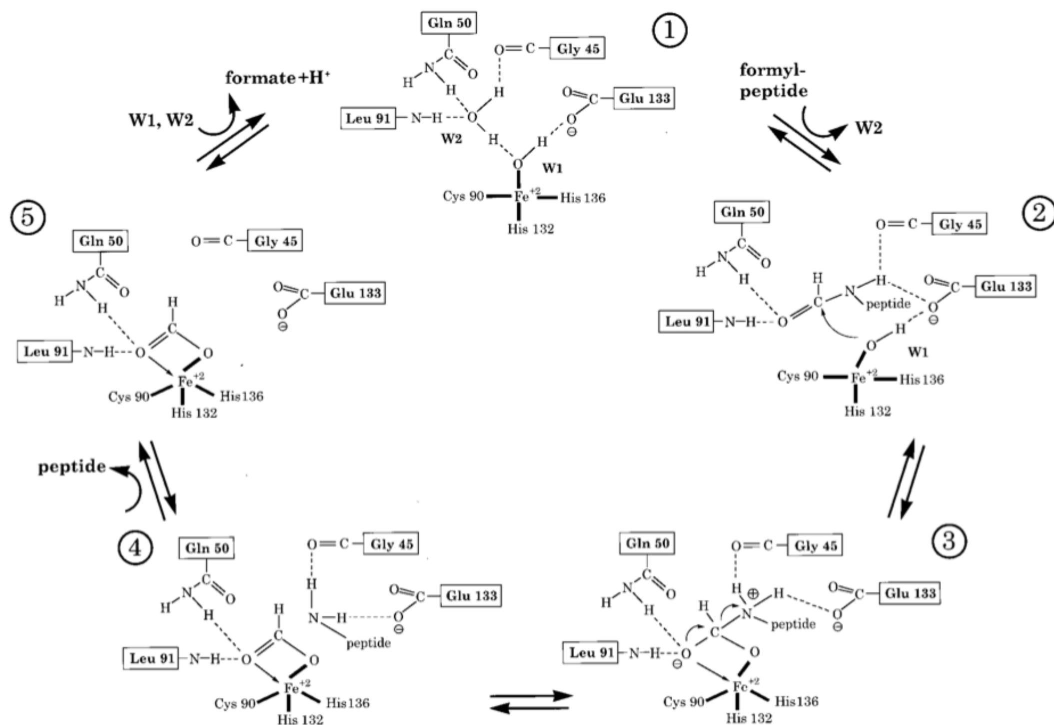


Figure 1.2. Proposed reaction mechanism of iron-metallated peptide deformylase, highlighting the role of metals in mononuclear enzymes [9]. The metal provides geometric flexibility by changing its coordination environment, as the reaction proceeds.

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CHAPTER 2: MANGANESE IMPORT IS A KEY ELEMENT OF THE OxyR RESPONSE TO HYDROGEN PEROXIDE IN *ESCHERICHIA COLI*

2.1 INTRODUCTION

To cope with the threat of reactive oxygen species (ROS), microbes have evolved inducible defensive regulons that are controlled by H_2O_2-sensing regulatory proteins—OxyR [12] and PerR [36] in bacteria, and Yap-1 [45] in yeast. In *E. coli*, intracellular H_2O_2 stress activates the OxyR transcription factor, which then directly stimulates synthesis of approximately two-dozen proteins [65]. The protective roles of about half of these are understood. The AhpCF peroxidase and the KatG catalase directly limit H_2O_2 accumulation [53]. Dps is a ferritin-like protein that sequesters unincorporated iron and thereby minimizes Fenton chemistry and, consequently, DNA damage [17, 23, 46]. The elevated expression of Fur protein, a repressor of iron-import genes, further limits the amount of intracellular iron [60, 64]. The *suf* genes encode an iron-sulfur-cluster assembly complex that replaces the customary Isc machinery [35, 56], which evidently is also poisoned by H_2O_2 (Jang and Imlay, in preparation).

The roles of the other OxyR-controlled genes are not yet known. One of these is *mntH*, which encodes the only dedicated manganese importer in *E. coli* [30]. Studies in a variety of other microbes have indicated that manganese can help protect cells against oxidative stress. Manganese supplementation allowed *Streptococcus pneumoniae* [58] and *Neisseria gonorrhoeae* [55] to better tolerate millimolar levels of H_2O_2 that might otherwise have been lethal. *Deinococcus radiodurans*, which is exceptionally resistant to ionizing radiation, lost a large component of that resistance when it was cultured in medium that limited the availability of manganese [14].

The basis of these protective effects is not clear. Manganese is an essential cofactor of manganese-containing superoxide dismutase, but this enzyme is not co-regulated by OxyR, as one might expect it to be if the intended effect of *mntH*

induction were to charge this enzyme. Instead, many workers have proposed that manganese might mitigate oxidative stress by chemically scavenging superoxide and/or hydrogen peroxide. Manganese complexes exhibit these activities *in vitro* [8, 19, 22], although the rate constants depend upon their coordinating ligands and, in the best cases, are still far lower than those of manganese-dependent superoxide dismutases and Mn-catalases.

The goal of this study was to inspect the role of MntH generally and during oxidative stress in particular. To do so we exploited strains of *E. coli* that lack catalases and peroxidases (Hpx^-). When cultured aerobically, these strains accumulate up to 1 μM of intracellular (and extracellular) H_2O_2 [54], a constant stress that moderately exceeds the dose (ca. 0.1 μM) that activates expression of the OxyR regulon [6]. We show here that in this situation a failure to induce MntH results in a profound growth defect. However, our analysis suggests that the requirement for manganese import stems from a need to metallate enzymes rather than to act as a chemical scavenger of ROS.

2.2 RESULTS

2.2.1 Manganese import into unstressed cells is minimal and expendable

E. coli has two cytoplasmic superoxide dismutases (SODs): an iron-dependent isozyme (FeSOD, encoded by *sodB*) [63] and a manganese-dependent isozyme (MnSOD, encoded by *sodA*) [27]. Activity-gel experiments by Pugh *et al.* indicated that MnSOD activity was elevated by an order of magnitude when manganese was added to standard defined growth medium [49]. This result could not be explained by the known mechanisms of *sodA* regulation, and it led workers to suggest that under routine growth conditions cells may not contain enough manganese to activate the apoenzyme. Using *sodB* mutants that express only the MnSOD isozyme, we confirmed that MnSOD activity rose from 2 U/mg to 40 U/mg when cells were grown in manganese-supplemented medium. A *sodA::lacZ* transcriptional fusion was not

induced by this treatment, as β-galactosidase activity was 3.2 +/- 0.06 U/mg under routine conditions and 2.9 +/- 0.04 U/mg after manganese supplementation.

To test the enzyme-activation idea directly, cell extracts were prepared from *sodB* mutants and assayed immediately in order to quantify MnSOD activity. The extracts were then treated with denaturants, demetallated with the chelators EDTA and 8-hydroxyquinoline-5-sulfonic acid, and then renatured in the presence of manganese in order to fully activate the MnSOD proteins. The denaturation/renaturation protocol boosted the initial extract activity by at least twenty-fold, indicating that < 5% of the enzyme molecules recovered from cells contained manganese in their active sites (Figure 2.1A, bars 1 and 2). In contrast, 60% of the MnSOD was already active when it was recovered from manganese-supplemented cells (Figure 2.1A, bars 3 and 4). Similar results were obtained with AB1157 and W3110 derivatives of *E. coli* K-12 (data not shown).

MnSOD activity in unsupplemented cells was boosted about six-fold when *mntH* was provided (behind its own promoter) on a multicopy plasmid (Figure 2.1A, bars 5 and 6). Thus the failure to fully metallate MnSOD *in vivo* derives from inadequate expression of the manganese importer. Several labs have shown that when MnSOD is recovered from iron-rich cells, a significant fraction contains iron and is therefore inactive [9, 61]. The preceding experiments were conducted at low cell densities in iron-sufficient medium, raising the possibility that inadequate manganese uptake allowed iron to outcompete manganese for binding to the apo-protein. However, follow-up experiments failed to distinguish whether the inactive MnSOD proteins contained iron or lacked metals entirely.

Reciprocal experiments with *sodA* mutants were performed to test the metallation status of FeSOD. Less than a 10% increase in activity was achieved by demetallation and remetallation with ferrous iron, indicating that the as-isolated FeSOD enzymes were fully loaded with iron. Collectively, these data indicate that when wild-type cells were cultured in standard media, they contained sufficient iron to fully metallate newly synthesized apo-FeSOD, while intracellular manganese was too scarce to effectively metallate apo-MnSOD. Indeed, inductively coupled plasma

(ICP) measurements indicated that the total intracellular manganese concentration was only 15 μM (Figure 2.2), compared to nearly 1 mM for iron ([44]; data not shown).

These results also suggest that *E. coli* can thrive even when it does not import enough manganese to metallate manganese-dependent enzymes. To more rigorously test this conclusion, we deleted the gene that encodes the only specific manganese transporter in *E. coli*, MntH. The manganese content of these mutants was minimal (Figure 2.2), and MnSOD activity was virtually absent (Figure 2.1, bars 7 and 8). Nevertheless, growth was as robust as that of wild-type cells (Figure 2.1B).

2.2.2 Cells require manganese import during H_2O_2 stress

These results raise the question: Under what circumstances is manganese import important for cell fitness? The regulatory proteins that control MntH synthesis may provide clues. One of these proteins, Fur, directly senses iron levels in *E. coli*. When iron is abundant, Fe^{2+}-metallated Fur protein binds the promoter region of *mntH* and represses its transcription [30, 47]. However, in low-iron conditions Fur is predominately in the apo-form, cannot bind DNA, and no longer inhibits *mntH* transcription. Thus the cell activates manganese import when iron levels are low. Such regulation implies that manganese can compensate for iron deficiency. Indeed, Grass *et al.* have shown that manganese entry through *mntH* can stimulate the growth of iron-import mutants [18]. We replicated these results (data not shown).

A plausible mechanism for this compensation is by the insertion of manganese into enzymes that normally employ iron as a cofactor. Manganese is unlikely to substitute for iron in iron-sulfur clusters or hemes, since the large disparity between its reduction potential and that of iron would interfere with the redox activities of these cofactors. Instead, it is possible that manganese takes the place of mononuclear iron in enzymes that employ divalent metals to bind substrates. *In vitro* a variety of metals, including manganese, typically suffice to activate these enzymes. The ability of iron to activate such enzymes has not been well explored. However, recent studies

show that enzymes such as isocitrate dehydrogenase [41] and transketolase (Sobota and Imlay, unpublished), which are commonly activated by manganese *in vitro*, are also fully functional if they are charged with ferrous iron. This raises the possibility that iron may be the usual cofactor for some metal-activated enzymes *in vivo*. If so, then during periods of iron scarcity the induction of MntH may enable manganese to may take its place. This substitution might also spare enough iron to charge those enzymes that require heme and iron-sulfur cofactors.

Another transcription factor, OxyR, induces expression of *mntH* during periods of H_2O_2 stress [28, 30]. The roles of OxyR-controlled genes have been studied in catalase/peroxidase (Hpx^-) mutants. These strains cannot degrade hydrogen peroxide; therefore, when they are cultured in aerobic media, the adventitious oxidation of intracellular redox enzymes leads to the accumulation of up to 1 μM intracellular H_2O_2 [54]. The OxyR stress response is activated by as little as 0.1 μM H_2O_2 [6], and we confirmed that an *mntH::lacZ* fusion was strongly induced in the Hpx^- mutant when it was transferred from anaerobic to aerobic media (Figure 2.3). In contrast, very little expression occurred in a wild-type background, consistent with the low level of intracellular manganese and the absence of a null-mutant phenotype.

A deletion mutation of *mntH* was generated in anaerobic Hpx^- mutants, without any apparent effect on anaerobic growth (data not shown). However, when the Hpx^- *ΔmntH* mutant strains were diluted into aerobic defined medium, they soon stopped growing (Figure 2.4A). This phenotype was reproduced in other *E. coli* K-12 backgrounds and in various types of defined media (data not shown). A plasmid carrying *mntH* restored normal growth, as did either the *katG* or *ahp* wild-type alleles. The addition of micromolar concentrations of manganese to the medium also allowed growth—while 100 μM iron, zinc, and copper did not (data not shown)—confirming that the pertinent role of MntH is to import manganese.

This result also indicates that there must be a secondary, low-affinity route by which manganese can enter the cell (Figure 2.1A, bars 7-10). That route is

unidentified but required 50 μM manganese for full growth stimulation, suggesting that its native substrate may be a cation other than manganese. In contrast, MntH imported sufficient manganese from the standard defined medium, which contained only 0.14 +/- 0.01 μM manganese. Interestingly, the Hpx^- *ΔmntH* mutants were able to grow in unsupplemented LB medium, apparently because they accumulated substantial intracellular manganese (Figure 2.2). LB medium contains more manganese (0.25 +/- 0.01 μM manganese) than does defined medium, and unlike the defined medium it lacks phosphate and citrate, metal chelators that can restrict manganese availability.

In the Hpx^- mutants the impact of the OxyR-dependent induction of *mntH* was to elevate the intracellular manganese content to 150 μM, an order of magnitude above that of wild-type cells (Figure 2.2). As a consequence, the efficiency of MnSOD activation was much higher (Figure 2.4B).

We deleted the OxyR binding site upstream of *mntH*. This mutation did not diminish basal expression, but it eliminated the OxyR-driven induction of the gene during H_2O_2 stress (data not shown). The Hpx^- mutants containing this non-inducible allele exhibited a growth behavior intermediate between that of the Hpx^- *ΔmntH* strain and its *mntH*$^+$ parent (Figure 2.4A). Thus basal expression of *mntH* is inadequate for H_2O_2-stressed cells, presumably because they require supranormal levels of manganese. Indeed, even the Hpx^- (*mntH*$^+$) strain grew best when additional manganese was added to the medium (data not shown).

2.2.3 Manganese import is not required to scavenge reactive oxygen species

Previous reports speculated that manganese might protect oxidatively stressed cells because it has the chemical ability to scavenge superoxide and H_2O_2 [8, 19, 22]. The Hpx^- strain suffers specifically from H_2O_2 stress, and so we directly measured the rates at which Hpx^- *mntH*$^+$ and Hpx^- *ΔmntH* cells degraded H_2O_2 (Figure 2.5A). Contrary to the scavenging hypothesis, the $MntH^+$ cells did not degrade H_2O_2 any

more rapidly than did their *ΔmntH* counterparts. Further, manganese supplements did not provide any activity. More generally, Hpx^- *mntH*$^+$ cells released as much H_2O_2 as did Hpx^- *ΔmntH* mutants (Figure 2.5B). Since the rate of efflux is directly proportional to internal H_2O_2 concentrations [54], this result indicates that imported manganese did not diminish intracellular H_2O_2 levels.

Superoxide degrades the iron-sulfur clusters of dehydratases, and the released iron can catalyze DNA damage by H_2O_2 [16, 31, 34, 37]. This mechanism provides a route by which superoxide can exacerbate the toxicity of H_2O_2; therefore, we examined the possibility that imported manganese was needed to suppress Fenton chemistry by activating MnSOD. However, a Hpx^- *ΔsodA* mutant grew well in defined medium, indicating both that MnSOD activity was expendable and that imported manganese was critical for some other purpose (Figure 2.6A). In a complementary experiment, the *sodA* gene was provided on a multicopy plasmid to boost the SOD activity of Hpx^- *ΔmntH* cells. Still, the cells were able to grow in aerobic medium only if manganese supplements were provided (Figure 2.6B). Indeed, when the cells were washed free of the manganese, growth quickly ceased even though the residual SOD activity substantially exceeded that of a wild-type strain. Most tellingly, the *sodA*-overexpressing plasmid actually inhibited the aerobic growth of an Hpx^- strain that contained the wild-type *mntH* allele—a strain which otherwise would have grown well—presumably because the elevated level of MnSOD protein sequestered the intracellular manganese so that it could not perform its critical (unknown) function. Finally, the addition of both *ΔsodA* and *ΔsodB* alleles to the Hpx^- strain did not block its growth in this medium (data not shown). All together, these results indicate that Hpx^- growth depends critically upon intracellular manganese rather than upon superoxide scavenging. We conclude that the key role of manganese in these H_2O_2-stressed cells is something other than the degradation of H_2O_2 or O_2^-.

2.2.4 Manganese does not protect DNA from oxidative damage

A primary target of H_2O_2 is DNA, through Fenton chemistry catalyzed by DNA-bound iron. We evaluated the possibility that manganese might protect the DNA by displacing iron from its surface. The 150 μM manganese that accumulated inside the Hpx^- cells was much less than the DNA nucleotide concentration in growing cells (15-30 mM) [42], indicating that manganese could never shield the DNA from all potential iron binding. Nevertheless, some DNA sequences create strong metal-binding sites and might be particular loci for damage [50], and in principle manganese might displace iron from these sites.

However, microscopic examination showed that Hpx^- *ΔmntH* cells did not filament, and despite their poor growth the number of viable cells did not diminish (data not shown). Filamentation and death are two hallmarks of oxidative DNA damage [46]. Further, although ICP analysis confirmed that high-dose manganese supplements raise the manganese content of wild-type cells to about 200 μM, these supplements did not diminish the H_2O_2 sensitivity of DNA-repair-defective mutants: only 1% of *recA* mutants survived a five-minute exposure to 2.5 mM H_2O_2 whether or not the growth medium was supplemented with 100 μM manganese. Finally, manganese supplements did not allow the growth of Hpx^- *ΔrecA* cells (data not shown). These latter experiments indicate that excess manganese does not suppress Fenton-mediated DNA damage. We infer that the growth defect of Hpx^- *ΔmntH* mutants does not arise from DNA oxidation and that it is more likely to reflect a disruption in cell metabolism.

2.2.5 Manganese is required by the cell to protect enzymes

When Hpx^- *ΔmntH* cells were supplemented with manganese, the amount of manganese that was needed to stimulate growth correlated with the amount needed to metallate MnSOD (Figure 2.7). Because MnSOD activity per se is not necessary to

allow growth, we interpret this correspondance more generally as an indication that H_2O_2-stressed cells need to import enough manganese to ensure metallation of one or more key enzymes. Aside from MnSOD, there are few or no enzymes in *E. coli* that specifically require manganese for function [29]. While many enzymes from *E. coli* can utilize manganese *in vitro* to satisfy a general requirement for a divalent metal, it is likely that many of these enzymes can use magnesium as an alternative. We knocked out several genes (*nrdEF, icd, pgmI, mntR, glpX, maeA*) that encode proteins that have been suspected of relying on manganese *in vivo*, but none of these mutations replicated the *ΔmntH* growth defect in the Hpx^- background (data not shown).

It is notable that magnesium binds more weakly than manganese to transketolase A and isocitrate dehydrogenase, perhaps because magnesium is less tolerant of deviations from its ideal coordination geometry [29]. If magnesium is a mediocre metal for such enzymes, and manganese is not imported into unstressed cells, then ferrous iron is a plausible candidate to be the usual activating metal. This logic suggests that manganese might serve to metallate such enzymes only during periods of iron starvation or H_2O_2 stress, the two conditions that activate synthesis of MntH.

During H_2O_2 stress the OxyR system also induces Dps, a ferritin-like protein that sequesters loose (free) iron and thereby diminishes the amount of Fenton-based DNA damage [17, 23, 46]. Therefore, one possible rationale for *mntH* induction during H_2O_2 stress would be that the action of Dps effectively starves cells for iron, so that without manganese the divalent-metal-requiring enzymes would be left in an apoprotein form. An alternative hypothesis is that ferrous-iron-loaded enzymes would be targets for Fenton reaction, and that the replacement of iron by manganese allows these enzymes to avoid oxidative injury. A testable difference between these two ideas is that the first hypothesis suggests that manganese protects the cell when it has too little unincorporated iron, whereas the second implies that manganese protects the cell when it has too much.

EPR-based measurements indicated that Hpx^- mutants contain more unincorporated iron than do wild-type cells [46]. The increase is likely due to the oxidative degradation of iron-sulfur clusters [26]; in any case, this result is more consistent with the second hypothesis rather than the first. Further, we observed that the profound growth defect of Hpx^- *Δdps* mutants could be suppressed by manganese supplements (Figure 2.8A). This result, too, suggests that manganese rescues the cell from iron overload rather than from iron starvation. Finally, iron chelators substantially stimulated the growth of Hpx^- *ΔmntH* cells (Figure 2.8B), indicating that the purpose of manganese is to out-compete iron.

These results supported the notion that manganese incorporation might spare enzymes from iron-driven oxidation reactions. Some fraction of Fenton events within proteins generate carbonyl groups on local amino acid residues. These can be detected on Western blots using antibodies against the DNP-derivatized proteins. Proteins extracted from Hpx^- *ΔmntH* mutants exhibited substantially more carbonyls than did proteins from their $MntH^+$ parents (Figure 2.9). The excess carbonylation was suppressed when manganese was supplemented into their growth medium. Further, the Hpx^- *Δdps* mutants exhibited extremely high levels of carbonylation, and this damage was also diminished by manganese. These data confirm that excess intracellular iron accelerates protein oxidation, while manganese import suppresses it.

2.3 DISCUSSION

2.3.1 Are manganese and iron alternative cofactors for mononuclear-metal enzymes?

Life evolved in an anaerobic, iron-rich world. Photosystem II did not begin to generate molecular oxygen until about 2.8 byr ago, and it is likely that during the subsequent billion years the atmosphere still remained essentially anaerobic, as the nascent oxygen was quickly scavenged by reaction with dissolved ferrous iron in the Earth's seas [11]. Only later, after the iron had been oxidized and precipitated as

ferric minerals, did oxygen accumulate. Thus the fundamental biochemical mechanisms and metabolic pathways that contemporary organisms use were inherited from an environment very unlike our own.

The depletion of bioavailable iron posed a problem for organisms, and they responded by inventing elaborate import systems to satisfy their iron demand. *E. coli* grown in laboratory cultures contains about 1 mM iron [44], much of it incorporated as a cofactor in enzymes that rely upon heme or iron-sulfur clusters. However, the fact that manganese import is stimulated upon iron depletion [30, 47], and that it can substantially compensate for an insufficient iron supply [18], suggests that a substantial fraction of imported iron might be used as a mononuclear cofactor in non-redox enzymes and that manganese can substitute for it. Indeed, although more than sixty *E. coli* enzymes can be activated by manganese *in vitro* (www.ecocyc.org), the robust growth of *mntH* mutants shows that these enzymes must be able to employ some other metal *in vivo*. Some of these enzymes are likely to use magnesium, but for others iron is the obvious candidate, since in its ferrous form it shares with manganese(II) a similar size, valence, and coordination geometry. Most manganese-activatable enzymes have not been adequately tested for their ability to use iron, because when such experiments are performed in aerobic buffers, ferrous iron is quickly oxidized to the unusable ferric form. We suspect, then, that some of these proteins evolved with iron as their routine cofactor, and that metallation by manganese is an alternative that is resorted to only when iron levels are low.

The few mononuclear enzymes that have redox functions may be instructive exceptions. For example, although iron and manganese again compete to bind in the protein coordination environments that are presented by superoxide dismutases, the two metals cannot provide equivalent activity, since they differ in their reduction potentials. To cope with this issue, discrete SOD isozymes are synthesized in *E. coli* and in many other bacteria, one for iron and one for manganese [27, 63]. Under iron-sufficient conditions, the iron-dependent isozyme is synthesized and the manganese-dependent enzyme is substantially repressed [57], suggesting that the latter is a back-up enzyme used in circumstances of iron deficiency.

Certain organisms seem to have evolved to not depend on iron for cell chemistry, including *Lactobacillus plantarum* [3], *Borrelia burgdorfei* [48] and *Streptococcus suis* [43]; notably, they have unusually high concentrations of intracellular manganese, which might serve as a cofactor for mononuclear metal enzymes. These bacteria additionally dispense with pathways that depend upon Fe/S- or heme-cofactored enzymes, such as the TCA cycle, and/or employ manganese-based enzymes in their place. For instance, *L. plantarum* uses a heme-less Mn-catalase to scavenge H_2O_2 [7, 33].

2.3.2 Manganese is not an efficient scavenger of ROS

The ability of manganese to defend microbes against oxidants has been noted in several contexts: imported manganese protects *Neisseria gonorrhoeae* [55, 59], *Streptococcus pneumoniae* [58], and *Salmonella typhimurium* [30] against exogenous H_2O_2, while lactic-acid bacteria, which generate H_2O_2 as a stoichiometic by-product of central metabolism, are rich in intracellular manganese [3]. Further, *Deinococcus radiodurans* loses its characteristic resistance to ionizing radiation if manganese import is limited [14]. Collectively these results have prompted suggestions that manganese might serve inside the cell as a chemical scavenger of superoxide [2, 4, 13, 14, 21, 25, 52, 59], of hydrogen peroxide [51], or of both [22, 29, 30, 55]. However, the peroxide-efflux experiments reported here directly show that in *E. coli*, at least, the imported manganese does not effectively degrade H_2O_2. Why not? Stadtman and colleagues showed that the ability of manganese to rapidly disproportionate H_2O_2 requires the formation of a manganese complex with three equivalents of HCO_3^- [8]. Metabolites are likely to interfere with this activity by coordinating the metal; and, even if they did not, the rate constant of the dismutation reaction is too low to enable physiological concentrations of manganese to effectively degrade H_2O_2. For example, if intracellular bicarbonate were, generously, 28 mM and manganese were 150 μM, the half-life of H_2O_2 would be 27 min, exclusive of the activities of Ahp and catalase. When the latter are considered, the half-life would be 1.3 msec (calculated from [54]). In other words, the scavenging activities of these

enzymes exceed the chemical activity of this concentration of manganese by six orders of magnitude. This calculation explains why imported manganese did not alter H_2O_2 efflux, and it confirms that the role of MntH induction is not to augment the scavenging activity of Ahp and KatG. It also suggests why *L. plantarum*, which has a high intracellular content of manganese, nevertheless synthesizes a dedicated Mn-catalase.

What about superoxide dismutation? This reaction can be accomplished by manganese that is loosely bound to certain biomolecules, with rate constants that reached 13,200 SOD units/mg Mn(II) in the best case [5]. In a cell containing 150 μM manganese, this ideal complex would provide 108 U/mL of dismutation activity. That amount still pales, however, compared to the 3000 U/mL enzymatic SOD present in unstressed cells [24] and the 14,000 U/mL in H_2O_2-stressed *E. coli* (calculated from this study). For both superoxide and H_2O_2 dismutation reactions, the kinetic efficiency depends upon the midpoint potential of the Mn(II)/Mn(III) couple and therefore is strongly affected by the coordination environment. The purpose of SOD and catalase proteins is to control that environment. So it seems unlikely that cells would rely on manganese that is loosely deposited on the surfaces of various biomolecules to serve this catalytic role. Interestingly, while there has been great interest in the use of manganese chelates as therapeutic sources of SOD activity, experiments have indicated that these drugs may not work by scavenging superoxide [40]. One alternative is that they serve as cell-permeable carriers of manganese, which then acts to metallate and protect mononuclear metal enzymes.

2.3.3 The OxyR regulon is dedicated to the suppression of Fenton chemistry

The OxyR regulon has been calibrated by evolution to be activated by about 0.1 μM intracellular H_2O_2, suggesting that this dose is physiologically relevant [6]. Because the cell membrane is only semi-permeable to hydrogen peroxide, an extracellular concentration of 1 to 5 μM H_2O_2 is probably what activates this system inside a wild-type (scavenger-proficient) cell [54]; therefore, this is the concentration

that one would like to impose in order to study the physiology of oxidative stress. However, it is difficult to establish and maintain this dose in experimental cultures of substantial cell density: wild-type cells rapidly degrade H_2O_2 that is provided in a single bolus (e.g., Figure 2.5A), and it is not easy to arrange continuous H_2O_2 production in order to replicate the steady-state H_2O_2 levels that might occur in nature. As an alternative, catalase/peroxidase-deficient (Hpx^-) strains permit workers to impose steady, physiological H_2O_2 stresses for a long enough period of time so that growth defects can become apparent.

To date the debilitating effects of micromolar H_2O_2 stress have all been tracked to Fenton-type reactions, including the oxidation of DNA [46], the destruction of dehydratase iron-sulfur clusters [26], and the de-regulation of iron homeostasis through the inactivation of Fur protein [60]. The rate constant of the Fenton reaction ranges from 10^3-10^4 M^{-1} s^{-1} for these biological targets. Thus growth defects arise and mutagenesis becomes prominent when intracellular H_2O_2 concentrations reach 1 μM, and it is fitting that the OxyR protein becomes activated when H_2O_2 levels approach this value. The proteins whose synthesis it activates include Dps, which suppresses Fenton chemistry by sequestering unincorporated iron; Fur, which restores control of iron import; and the Suf system, which helps to maintain the activities of Fe/S enzymes. The determination that MntH also helps suppress Fenton-based protein damage fits this pattern. However, we have not yet identified the specific metabolic failure that causes either the Hpx^- *Δdps* mutant or the Hpx^- *ΔmntH* mutant to stop growing. The suspicion is that an iron-loaded mononuclear-metal enzyme is oxidized and loses activity, thereby plugging a key metabolic pathway. We are currently working to identify such a bottleneck.

2.4 EXPERIMENTAL PROCEDURES

2.4.1 Reagents

Desferrioxamine (deferoxamine mesylate), 30% hydrogen peroxide, o-nitrophenyl-β-D-galactopyranoside (ONPG), acid-hydrolyzed casein hydrolysate (Hy-Case Amino), xanthine, bovine xanthine oxidase, *E. coli* manganese-containing superoxide dismutase, horseradish peroxidase, *E. coli* iron-containing superoxide dismutase, horse heart cytochrome *c*, manganese(II) chloride tetrahydrate, copper(II) sulfate pentahydrate, ferric chloride, ferrous ammonium sulfate hexahydrate, zinc sulfate, and 8-hydroxyquinoline-5-sulfonic acid were from Sigma-Aldrich. Guanidine hydrochloride was obtained from Fisher Scientific. OxyBlot Protein Oxidation Detection Kit (S7150) was from Chemical International. Amplex Red was purchased from Invitrogen.

2.4.2 Bacterial growth

Luria-Bertani medium (LB) contained (per liter) 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl. Defined medium (glucose/amino acids) consisted of minimal A salts [39] supplemented with 0.2% casein hydrolysate, 0.5 mM tryptophan, and 0.2% glucose. When antibiotic selection was needed, media were supplemented with either 100 μg/mL ampicillin or 20 μg/mL chloramphenicol.

Anaerobic cultures were grown in an anaerobic chamber (Coy Laboratory Products Inc.) under an atmosphere of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. Aerobic cultures were grown with vigorous shaking at 37°C. To ensure that cells were growing exponentially before they were exposed to oxygen, anaerobic overnight cultures of oxygen-sensitive strains were diluted to OD_{600} = 0.005 in fresh anaerobic medium and allowed to grow to OD_{600} ~ 0.15 at 37°C. These cells were then subcultured into fresh aerobic medium to obtain an OD_{600} of 0.005, with or without manganese(II) chloride, and grown aerobically at 37°C. To remove

supplemented manganese, cells were washed once and inoculated in the same medium but without $MnCl_2$.

2.4.3 Strains and strain construction

Strains used in this study are listed in Table 1. All constructions in Hpx^- (i.e. *katE katG ahp*) backgrounds were performed in an anaerobic chamber to ensure that suppressor mutations were not selected during outgrowth. Null mutations were created using the Red recombinase method [15]. Mutations were introduced into new strains by P1 transduction [39]. The resultant mutations were confirmed by PCR analysis and, when possible, by enzyme assays.

The chromosomal *mntH(NI)* allele, in which the OxyR binding site is removed and replaced with a *flp* scar sequence, was also generated by the Red recombinase method. These mutations were confirmed by PCR analysis and sequencing of the PCR products. Positions –111 to –75 upstream of the transcription start site were removed in the *mntH(NI*) mutant allele, corresponding to bases 2510803 to 2510839. This deletion removed the OxyR binding site [28] but did not alter the -10/-35 promoter region or the 5' UTR of the transcript. The plasmid pAA01 expressing the wild-type *mntH* allele and its promoter region (base 2509490-base 2510928) was made using the forward primer 5'-ATCTAAAGCTTGCTATGTTGTGTATGGAAGCTGAAAG and reverse primer 5'-GAATCGGATCCCTACAATCCCAGCGCCGACCCCAC with BamHI and HindIII restriction sites. The PCR product was inserted into pBR322 plasmid and confirmed by sequencing.

Single-copy *lacZ* fusions to *sodA* and *mntH* promoter regions were integrated into the lambda attachment site, while the wild-type genes remained at their native positions [20]. The promoter regions were amplified using the forward primer 5'-GAGAGACTAAACGAGCTGTAATACGCC and reverse primer 5'-CATATTCATCTCCAGTATTGTCGGGCG for *sodA*, and forward primer 5'-ATATCCTGCAGCAACAACGGCAAGTGCCAGTACAAAATG and reverse

primer 5’-ATATCGGTACCCATCTTGTGCCTCTAAAACATAGCCTTTG for *mntH*, both designed with PstI and KpnI restriction sites. CRIM plasmids were modified by replacing the kanamycin-resistance cassette with a chloramphenicol-resistance cassette, to permit antibiotic selection under anaerobic conditions, and engineering FLP sites around the chloramphenicol acetyltransferase gene. The *sodA* and *mntH* promoter regions were inserted, and the resultant plasmid constructions were confirmed by sequencing. After chromosomal integration and transduction into recipient strains, the chloramphenicol acetyltransferase gene was removed from mutants, as indicated in table 1, by using the temperature sensitive plasmid pCP20, which was later cured from the strain in question [15].

2.4.4 Enzyme assays

Aerobic cultures were grown in defined medium (glucose/amino acids) to an OD_{600} of 0.2, after which cells were centrifuged, washed, resuspended, and sonicated in 50 mM potassium phosphate buffer (pH 7.8) with 0.1 mM EDTA. We measured β-galactosidase activity by ONPG hydrolysis [39], and determined total protein content by using the Coomassie blue dye-binding assay (Pierce). SOD activity was measured by the xanthine oxidase-cytochrome *c* method [38]. To track the activity of specific SOD isozymes, we measured manganese-containing SOD (MnSOD) in *sodB* mutants and iron-containing SOD (FeSOD) in *sodA* mutants. The fraction of each isozyme that was active in cell extracts was determined after extracts were subjected to partial denaturation and renaturation in the presence of manganese/iron to ensure the full activation of MnSOD or FeSOD proteins. MnSOD was denatured at pH 3.8 in the presence of guanidinium chloride, EDTA, and 8-hydroxyquinoline-5-sulfonic acid and then reactivated by dialysis into neutral pH in buffer containing 0.1 mM $MnCl_2$. Details follow the published protocol [32] except that 8-hydroxyquinoline-5-sulfonic acid was used instead of 8-hydroxyquinoline and remetallation was accomplished with two changes of 0.1 mM $MnCl_2$ in 5 mM Tris-HCl buffer at pH 7.8 for 12 h each instead of one. Metals were removed from FeSOD at pH 11 in the presence of EDTA and dithiothreitol, while remetallation was

accomplished by dialysis at neutral pH against 0.1 mM ferrous salts and dithiothreitol [62], all in an anaerobic chamber [10]. For both MnSOD and FeSOD reconstitution, purified MnSOD and FeSOD were used as controls; in both cases, all activity was lost upon demetallation and fully recovered by reconstitution. The periplasmic CuZnSOD is only synthesized in stationary phase, and activity gels confirmed that it did not provide detectable activity under the experimental conditions used here.

2.4.5 Inductively coupled plasma (ICP) measurement on intracellular manganese

One-liter cultures were grown aerobically in defined medium (glucose/amino acids) at 37°C to an OD_{600} of 0.15-0.2. The cells were then centrifuged, washed twice with 200 mL ice-cold 20 mM Tris-HCl/1mM EDTA (pH 7.4), washed once in the same buffer without EDTA, and then resuspended in 4 mL 20 mM Tris-HCl (pH 7.4). Cells were lysed with a French press, and debris was pelleted by centrifugation at 22,000 x *g* for 25 min. The metal content of the supernatant was determined with a SCIEX ELAN DRCe (Perkin-Elmer), and protein content was determined using the Coomassie blue dye-binding assay (Pierce). To calculate the manganese concentration of intact cells, the degree of dilution that occurred during lysis was deduced by comparing the protein concentration of the lysate to its known concentration in intact cells (~300 mg/mL, determined previously by isotopic labeling of the cytoplasmic volume, coupled with measurement of cellular protein [24]).

2.4.6 H_2O_2 concentration measurement

Cells were grown anaerobically in defined medium (glucose/amino acids) to an OD_{600} of ~0.15. These cells were then centrifuged, suspended in fresh defined medium at an OD_{600} of 0.005, and grown aerobically at 37°C with vigorous shaking, with or without 50 μM $MnCl_2$. At regular intervals an aliquot was removed and centrifuged, and H_2O_2 was measured in the supernatant. H_2O_2 was detected using the

Amplex Red/horseradish peroxidase method [53]. Fluorescence was then measured in a Shimadzu RF Mini-150 fluorometer.

The rates of H_2O_2 scavenging by whole cells were measured in a similar way, except cells were inoculated aerobically to an OD_{600} of 0.02, and 1 μM H_2O_2 was added to the culture.

2.4.7 Cell viability

Air-sensitive strains were grown anaerobically in defined medium (glucose/amino acids) to an OD_{600} of ~0.15. These cells were then subcultured to an OD_{600} of 0.003 and grown aerobically at 37°C with vigorous shaking. At intervals, aliquots of cells were moved to the anaerobic chamber, diluted in anaerobic LB, and plated on anaerobic LB agar containing 0.2% glucose. Colonies were enumerated after overnight anaerobic incubation at 37°C.

2.4.8 H_2O_2 killing assay

Cells were grown aerobically in defined medium (glucose/amino acids) to an OD_{600} of 0.1-0.15 with or without 100 μM $MnCl_2$ supplementation. These cells were subcultured to an OD_{600} of 0.025, with or without 100 μM $MnCl_2$ supplementation. H_2O_2 (2.5 mM) was added, and at different times aliquots were diluted with LB containing catalase, and colonies were enumerated on LB plates after overnight incubation at 37°C.

2.4.9 Protein carbonylation

Cells were grown anaerobically in defined medium (glucose/amino acids) at 37°C to an OD_{600} of 0.1-0.15. Most strains were diluted to an OD_{600} of 0.005 in freshly made aerobic defined medium (glucose/amino acids), with or without 50 μM

$MnCl_2$ supplementation. Hpx$^-$ *mntH* cells, grown without $MnCl_2$, were subcultured to an OD_{600} of 0.040, since these cells double in biomass only twice before growth ceases. Cells were harvested at an OD_{600} of ~ 0.2. These cells were washed twice with ice-cold 50 mM potassium phosphate buffer pH 7.0, after which they were suspended in 500 μL of the same buffer with the addition of 5 mM DETAPAC. DETAPAC prevents further protein oxidation in extracts. Cells were sonicated, and then protein carbonylation was measured using OxyBlot Protein Oxidation Detection Kit (S7150) (Chemicon International). Protein carbonyl groups were derivatized to 2,4-dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine (DNPH) for 15 min in 3% (w/v) SDS. β-Mercaptoethanol (1% v/v) was then added to these derivatized samples, after which the samples were subjected to polyacrylamide denaturing gel electrophoresis (4-15% BioRad). Proteins were then transferred to a nitrocellulose membrane (Amersham Hybond™-ECL™ by GE Healthcare) for 60 min at 100 V. The membrane was then incubated with primary antibody specific to the DNP moiety attached to the derivatized proteins. This step was followed by incubation with a horseradish peroxidase antibody conjugate directed against the primary antibody. The membranes were then treated with chemiluminescent substrate (GE Healthcare, Amersham ECL™ Western Blotting Analysis System) and imaged by exposure to light sensitive films (Amersham Hyperfilm ECL™ chemiluminescence film, GE Healthcare, Buckinghamshire, UK).

Some protein carbonyls were consistently detected in the extracts of cells than had been grown and processed under strictly anaerobic conditions. These carbonyl adducts constitute a background that cannot reflect ROS-mediated oxidative damage and may arise from protein glycation reactions [1]. This background must be considered when protein carbonylation is used as a proxy for oxidative injury.

2.5 TABLE

Table 2.1 Strain list

Strain	Genotype	Reference
MG1655	F$^-$ wild type	E. *coli* Genetic Stock Center
LC106	*ΔahpF::kan Δ*(*katG17*::Tn*10*)*1 Δ*(*katE12*::Tn*10*)*1*	Seaver and Imlay (2004)
CAG18467	*zff-1*::Tn*10*	E. *coli* Genetic Stock Center
MM2115	*mntH9*::*kan*	Kehres and Maguire (2000)
SMV41	*mntH9*::*kan~zff-1*::Tn*10*	P1(CAG18467) x MM2115
SMV42	As LC106 plus *mntH9*::*kan~zff-1*::Tn*10*	P1(SMV41) x LC106
BW25113	*lacI rrnB ΔlacZ hsdK ΔaraBAD ΔrhaBAD*	Datsenko and Wanner (2000)
AA19	BW25113 *ΔmntH2*::*cat*	This work
AA99	MG1655 *ΔmntH2*::*cat*	P1(AA19) x MG1655
AA30	LC106 *ΔmntH2*::*cat*	P1(AA19) xLC106
JS141	CSH7 *ΔahpCF1*::*cat Δ*(*katG17*::Tn*10*)*1 ΔkatE1*::*kan*	Laboratory Stock
AA118	JS141 *mntH9*::*kan~zff-1*::Tn*10*	P1(SMV41) x JS141
JS148	AB1157 *ΔahpCF1*::*cat Δ*(*katG17*::Tn*10*)*1 ΔkatE1*::*kan*	Laboratory Stock
AA120	JS148 *mntH9*::*kan~zff-1*::Tn*10*	P1(SMV41) x JS148
JI370	MG1655 *ΔahpF::kan*	Seaver and Imlay (2001)
AA116	JI370 *ΔmntH2*::*cat*	P1(AA19) x JI370
JI364	*Δ*(*katG17*::Tn*10*)*1*	Seaver and Imlay (2001)
AA142	JI364 *ΔmntH2*::*cat*	P1(AA19) x JI364
DH5α pir$^+$	*supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 pir$^+$*	Jim Slauch
SJ99	MG1655 *Δ*(*lacZ1*::*cat*)*1*	Laboratory Stock
G169	F$^-$, *aroD shiA proA argE zdg-299*::Tn*10*	Grogan and Cronan (1984)
JI132	AB1157 (*sodA*::*Mud* PR13)25 (*sodB*::*kan*)*1*-Δ2	Linn and Imlay (1987)
KK183	JI132 *zdg-299*::Tn*10*	P1(G169) x JI132
SMV29	LC106 (*sodB*::*kan*)*1*-Δ2	Varghese and Imlay (2007)
SMV32	MG1655(*sodB*::*kan*)*1*-Δ2	Varghese and Imlay (2007)
AA130	MG1655 *Δ*(*lacZ1*::*cat*)*1 attλ*::[pSJ501::*sodA'*-*lacZ*$^+$]	This work
AA138	SMV32 *Δ*(*lacZ1*::*cat*)*1 attλ*::[pSJ501::*sodA'*-*lacZ*$^+$]	P1(AA130) x AA136
AA114	SMV32 *ΔmntH2*::*cat*	P1(AA19) x SMV32
AA141	AA114 *Δ*(*lacZ1*::*cat*)*1 attλ*::[pSJ501::*sodA'*-*lacZ*$^+$]	P1(AA130) x AA137
KCI420	MG1655 (*sodA*::*Mud* PR13)*25*	Laboratory Stock
AA171	MG1655 *Δ*(*lacZ1*::*cat*)*1* attλ::[pSJ501::*mntH*'-*lacZ*$^+$]	This work
AA183	MG1655 *Δ*(*lacZ1*::*cat*)*1 attλ*::[pSJ501::*mntH'*-*lacZ*$^+$]	P1(AA171) x SJ130
SJ108	LC106 *Δ*(*lacZ1*::*cat*)*1*	Laboratory Stock
AA191	SJ108 *attλ*::[pSJ501::*mntH'*-*lacZ*$^+$]	P1(AA171) x SJ108
AA147	BW25113 *mntH1::cat(NI)*	This work
AA153	LC106 *mntH1*::*cat(NI)*	P1(AA147) x AA153

Table 2.1 (Continued)

SMV42/pAA01	SMV42 with pAA01	This work
AA145	SMV29 Δ*mntH2*::*cat*	P1(AA19) x SMV29
LC106/pDT1.5	LC106 with pDT1.5	This work
SMV42/pDT1.5	SMV42 with pDT1.5	This work
Lem17	MG1655 *recA56 srl300*::Tn*10*	Laboratory Stock
XY27	MG1655 plus *recA938*::*cat ΔahpF::kan Δ*(*katG17*::Tn*10*)*1 Δ*(*katE12*::Tn*10*)*1*	Park and Imlay (2005)
SP66	LC106 *mhpC281*::Tn*10 lacY1 dps*::*cat*	Park and Imlay (2005)
SMV30	LC106 (*sodA*::*Mud* PR13)*25*	P1(KK183) x LC106
JEM78	BW25113 *ΔnrdEF1*::*cat*	Julia Martin
JEM90	LC106 *ΔnrdEF1*::*cat*	P1(JEM78) x LC106
AA44	BW25113 *Δicd1*::*cat*	This work
AA160	LC106 *Δicd1*::*cat*	P1(AA44) x LC106
AA38	BW25113 *ΔpgmI1*::*cat*	This work
AA59	LC106 *ΔpgmI1*::*cat*	P1(AA38) x LC106
AA28	BW25113 *ΔmntR1*::*cat*	This work
AA33	LC106 *ΔmntR1*::*cat*	P1(AA28) x LC106
AA79	BW25113 *ΔglpX1*::*cat*	This work
AA89	LC106 *ΔglpX1*::*cat*	P1(AA89) x LC106
AA94	BW25113 *ΔmaeA1*::*cat*	This work
AA110	LC106 *ΔmaeA1*::*cat*	P1(AA94) x LC106

Plasmid	Genotype	Reference
pAA01	pBR322 with *mntH*	This work
pSJ501	pAH125 derivative with *cat* flanked by *flp* sites	Soojin Jang
pAA02	pSJ501::*sodA'*-*lacZ*$^{+}$	This work
pAA03	pSJ501::*mntH'*-*lacZ*$^{+}$	This work
pDT1.5	pBR322 with *sodA*	Carlioz and Touati (1986)

2.6 FIGURES

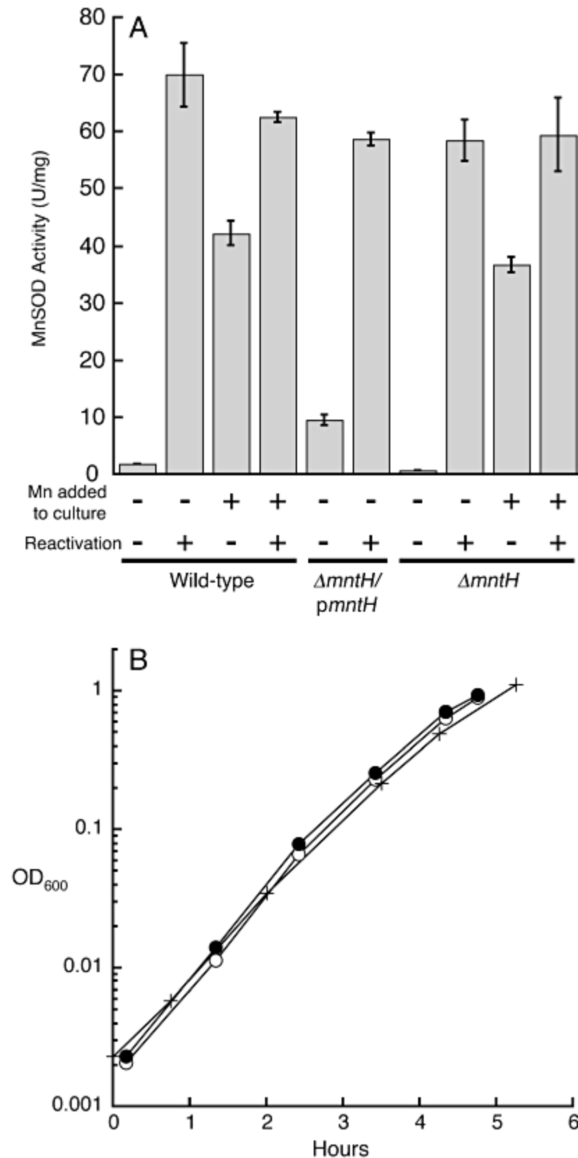

Figure 2.1. Unstressed cells do not depend upon manganese. A. In standard medium, manganese import is too slight to activate MnSOD. Where indicated, media were unsupplemented or were supplemented with 50 µM manganese, and cell extracts were assayed before and after *in vitro* demetallation/reactivation with manganese. Each strain lacks *sodB*, to permit assay of MnSOD. The strains were AA138 (*mntH*$^{+}$), AA141 (*ΔmntH*), and AA141/pAA01 (*ΔmntH/*p*mntH*). B. Manganese import is unnecessary for rapid growth. Wild-type MG1655 cultured without (open circles) or with (closed circles) 50 µM manganese supplement, and *ΔmntH* (AA99, plus sign) without manganese.

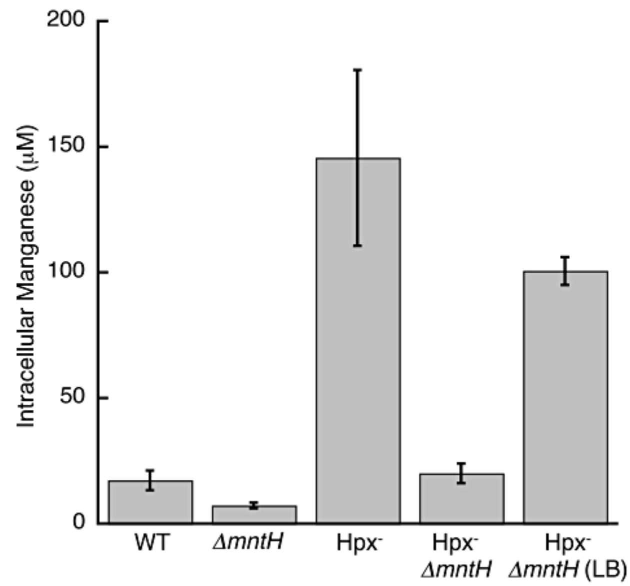

Figure 2.2. Total manganese concentration of cells, measured by ICP. Cells were grown in defined medium (glucose/amino acids) or, where indicated, LB medium. Data represents means of three independent cultures. The strains used were MG1655 (wild-type), AA99 (*ΔmntH*), LC106 (Hpx^-) and AA30 (Hpx^- *ΔmntH*).

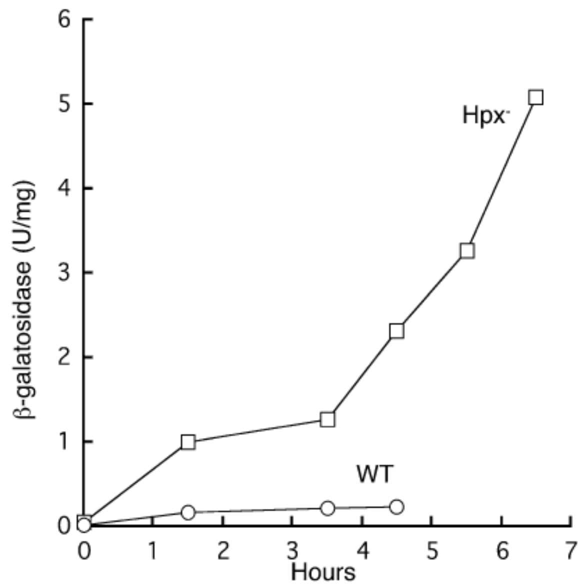

Figure 2.3. The gene *mntH* is strongly induced in aerobically growing Hpx$^-$ cells. Cells bearing a *mntH'-lacZ* fusion were grown in anaerobic defined medium (glucose/amino acids) and aerated at time zero. At intervals β-galactosidase was assayed. This experiment is representative of four independent replicates. AA183 (wild-type, circles) and AA191 (Hpx$^-$, squares).

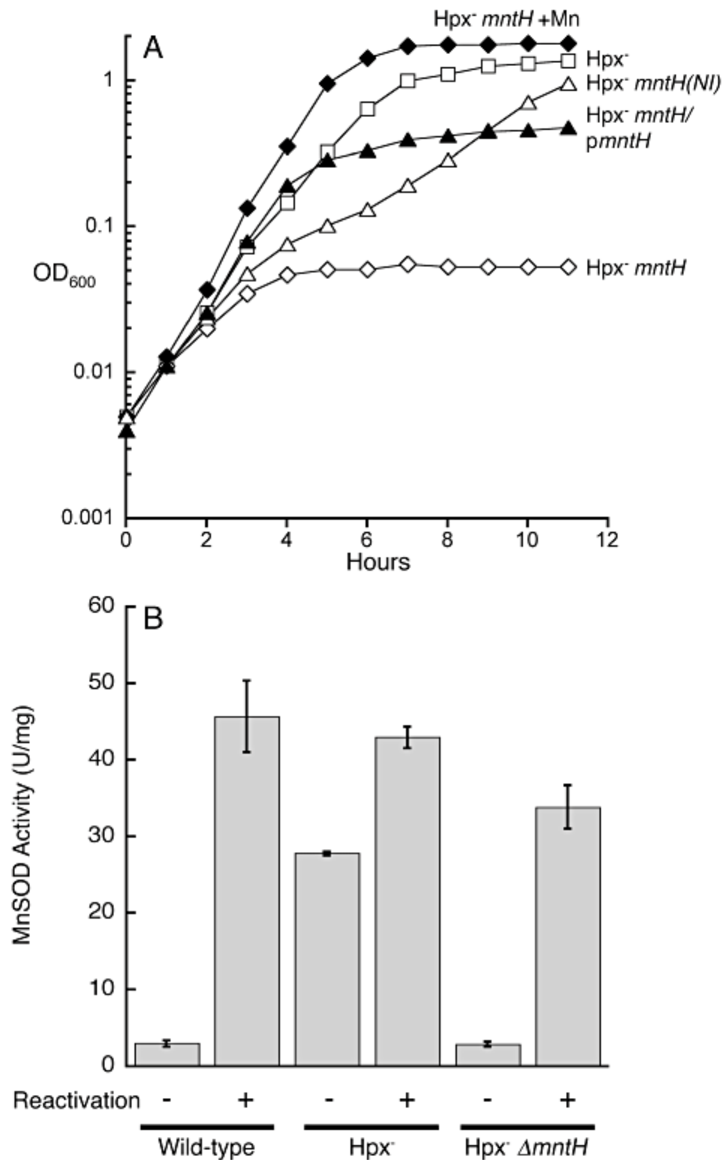

Figure 2.4. Manganese import is essential for H_2O_2-stressed cells. Cells were grown in defined medium (glucose/amino acids). A. Cells were cultured anaerobically and then aerated at time zero. Where indicated, manganese (50 μM) was included in the culture medium. LC106 (Hpx^-, open squares), SMV42 (Hpx^- *ΔmntH*, open diamonds), SMV42 + $MnCl_2$ (closed diamonds), SMV42/pAA01 (Hpx^- *ΔmntH*/p*mntH*, closed triangles) and AA153 (Hpx^- *mntH(NI)*, open triangles). B. MnSOD protein is correctly metallated in H_2O_2-stressed cells, due to increased manganese import. Each strain lacks *sodB*, to permit assay of MnSOD. SMV32 (wild-type), SMV29 (Hpx^-), AA145 (Hpx^- *ΔmntH*).

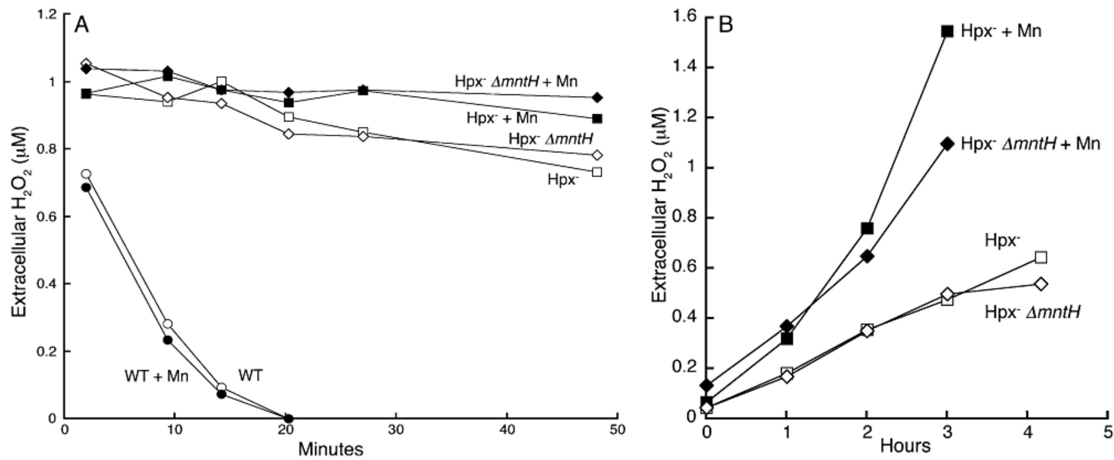

Figure 2.5. Imported manganese does not scavenge H_2O_2. A. Manganese did not prevent H_2O_2 accumulation by Hpx^- and Hpx^- *ΔmntH* cells. Cells were grown anaerobically and aerated at time zero. At intervals the medium was assayed for accumulated H_2O_2. LC106 (Hpx^-, squares) and SMV42 (Hpx^- *ΔmntH*, diamonds). Filled symbols: 50 μM manganese was included in the growth medium. B. Manganese-supplemented cells did not exhibit significant H_2O_2-scavenging activity. H_2O_2 (1 μM) was added at time zero to cultures of log-phase cells. Filled symbols: 50 μM manganese was included in the growth medium. MG1655 (open circles), LC106 (Hpx^-, squares), SMV42 (Hpx^- *ΔmntH*, diamonds).

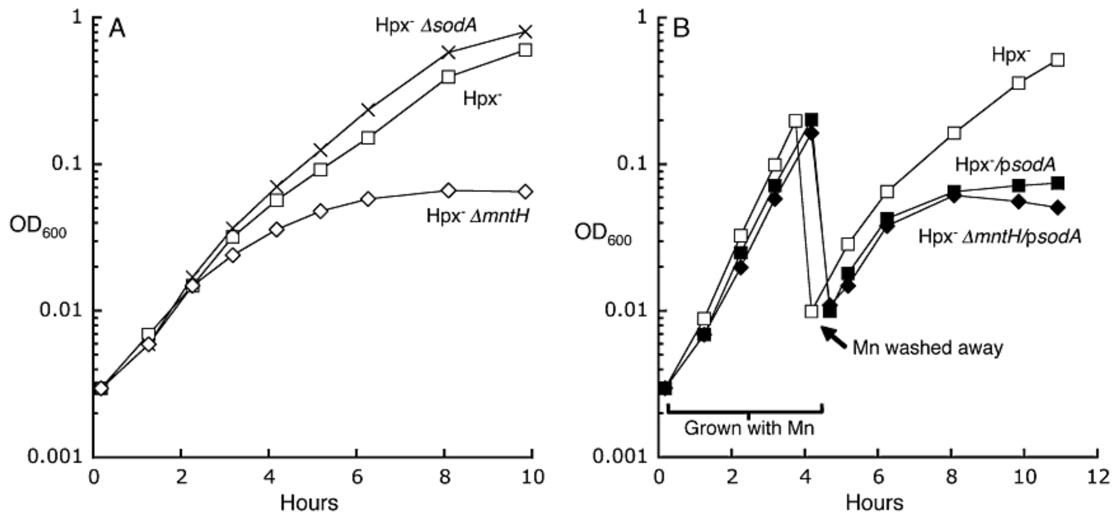

Figure 2.6. The primary role of intracellular manganese is not to degrade O_2^-. A. Metallation of MnSOD is not the primary purpose of manganese import. Cells were precultured in anaerobic defined medium (glucose/amino acids) and then aerated at time zero. LC106 (Hpx$^-$, squares), AA30 (Hpx$^-$ *ΔmntH*, diamonds) and SMV30 (Hpx$^-$ *ΔsodA*, X's). B. Overproduction of MnSOD debilitates Hpx$^-$ *ΔmntH* cells. Anaerobic cells, with or without the pDT1.5 *sodA* over-expression plasmid, were aerated at time zero in defined medium (glucose/amino acids) containing 100 μM $MnCl_2$. At the indicated time (arrow), manganese was removed. LC106 (Hpx$^-$, open squares), LC106/pDT1.5 (closed squares) and SMV42/pDT1.5 (Hpx$^-$ *ΔmntH*, closed diamonds).

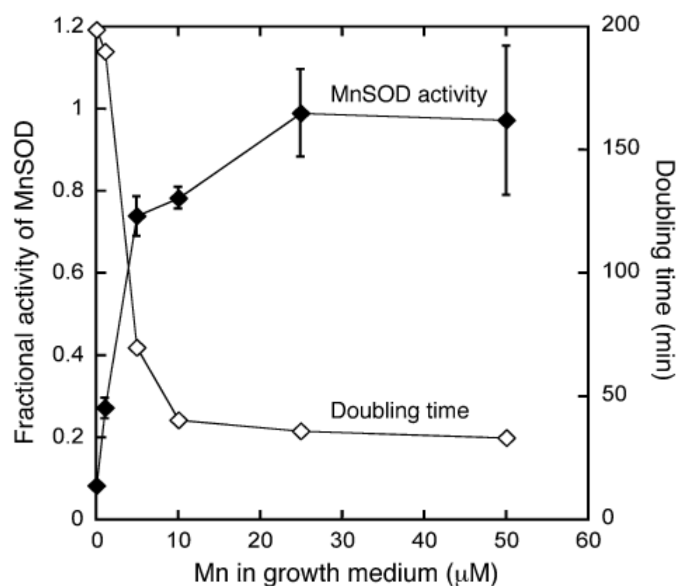

Figure 2.7. The amount of manganese needed to protect Hpx^- mutants matches the amount needed to metallate enzymes. AA145 (Hpx^- *ΔmntH*) cells were precultured anaerobically in defined medium (glucose/amino acids) and then subcultured at time zero into aerobic defined medium (glucose/amino acids) containing the indicated concentration of manganese. Optical density was monitored continuously, and MnSOD activity was measured when the cultures reached an OD_{600} of 0.2. The lowest doubling time, and thus the highest growth rate, for each culture is denoted in the figure.

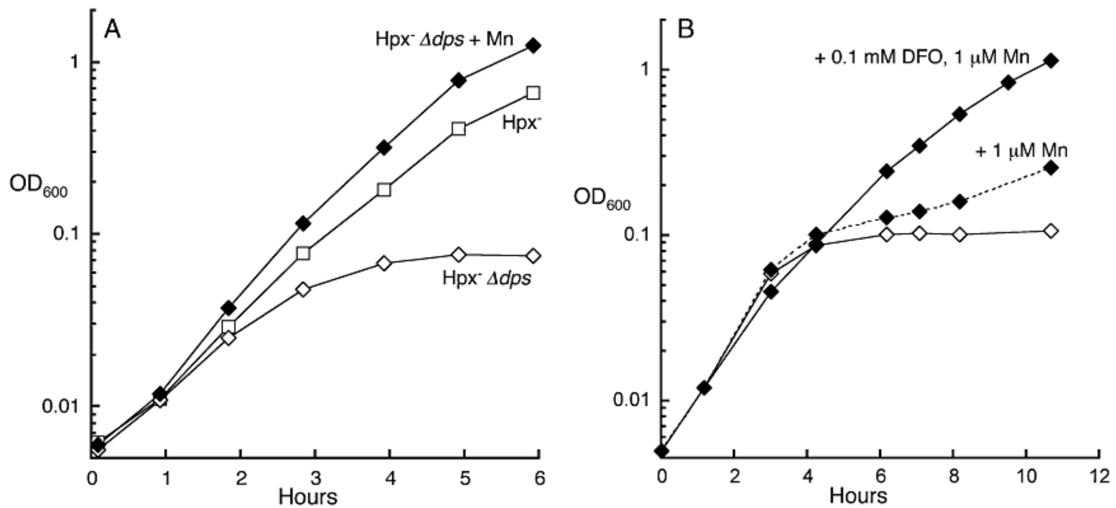

Figure 2.8. Manganese is required to rescue Hpx⁻ cells from iron overload. A. Manganese supplementation rescues Hpx⁻ *Δdps* cells. Cells growing in anaerobic defined medium (glucose/amino acids), +/- 50 μM $MnCl_2$, were aerated at time zero. LC106 (Hpx⁻, open squares), SP66 (Hpx⁻ *Δdps*, open diamonds), SP66 grown + $MnCl_2$ (closed diamonds). B. The growth defect of Hpx⁻ *ΔmntH* was suppressed by an iron-specific chelator. Cells (AA30) were grown in aerobic defined medium (glucose/amino acids) without $MnCl_2$ (open diamonds), with 1 μM $MnCl_2$ (dotted line, closed diamonds) or with 1 μM $MnCl_2$ plus 0.1 mM deferoxamine (closed diamonds).

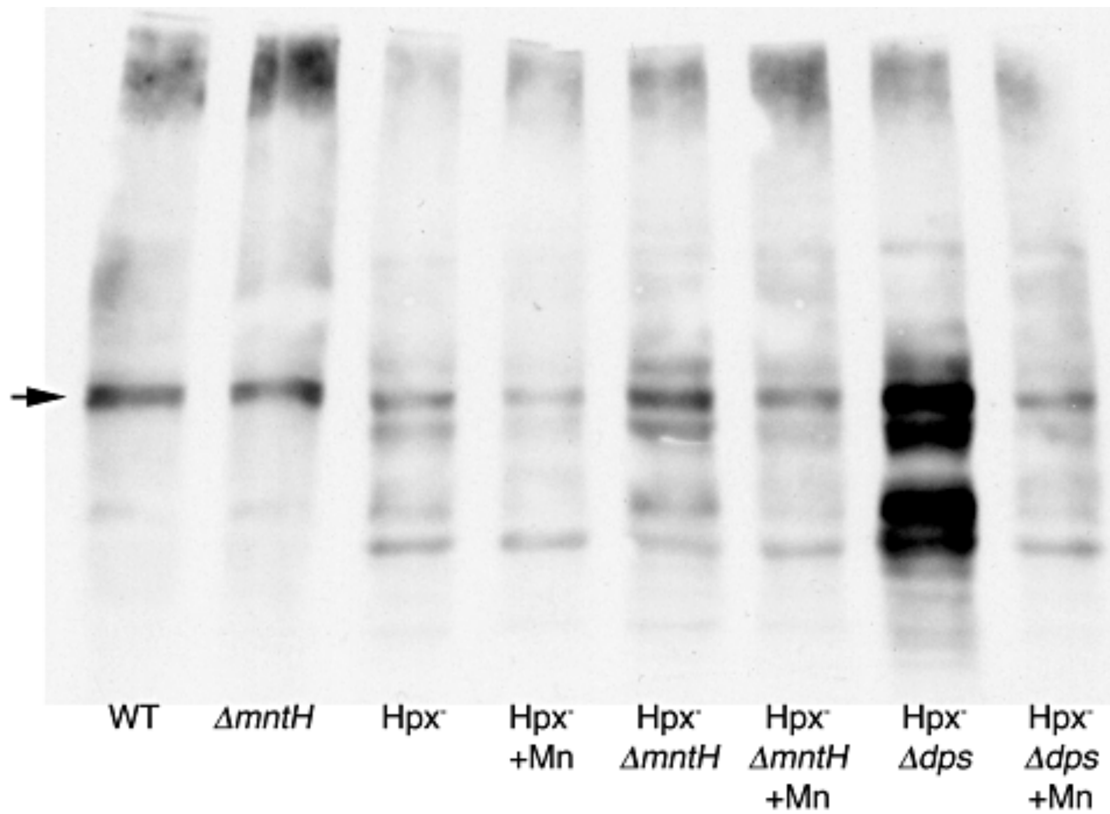

Figure 2.9. Imported manganese suppresses oxidative protein carbonylation. Cells were grown in aerobic defined medium (glucose/amino acids) +/- 50 μM $MnCl_2$, and proteins were harvested at an OD_{600} of ~0.2. Proteins were derivatized and western blotted as described in experimental procedures. The arrow indicates a non-oxidative carbonylation that was detected even when cells were grown and derivatized anaerobically. The strains used were MG1655 (wild-type), AA99 (*ΔmntH*), LC106 (Hpx^-), AA30 (Hpx^- *ΔmntH*) and SP66 (Hpx^- *Δdps*).

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CHAPTER 3: APO AND IRON-CHARGED MONONUCLEAR ENZYMES ARE RAPIDLY OXIDIZED BY H_2O_2, AND ARE PROTECTED BY MANGANESE

3.1 INTRODUCTION

We have previously shown that manganese import is critical in H_2O_2-stressed cells, since cells mutated for a manganese import system, *mntH*, stop growing when stressed with H_2O_2 [1]. In contrast, *mntH* mutants under routine growth conditions have no growth defect. The beneficial effect of manganese on H_2O_2-stressed cells has been known for quite some time, [6, 25, 29] and workers have speculated that the mechanism of manganese protection is by scavenging H_2O_2. We demonstrated that the requirement of manganese import during H_2O_2 stress is not to scavenge H_2O_2 [1]. Instead, we were able to demonstrate that manganese selectively protects oxidative damage to proteins [1]. We speculated that manganese must functionally replace iron in mononuclear enzymes, and thereby prevent Fenton-mediated damage to these proteins. In the present study we sought to identify such mononuclear enzymes that normally use iron as their cofactor, but during H_2O_2 stress are able to use manganese and thus prevent damage to these enzymes. *E. coli* is known to have over one hundred mononuclear enzymes (www.ecocyc.org). Considering that iron is the predominant transition metal in *E. coli* it is likely that quite a few of these enzymes use iron as their cofactor *in vivo*. We identified three such mononuclear enzymes, peptide deformylase, threonine dehydrogenase and cytosine deaminase, that are functionally distinct and yet use iron as their cofactor. We demonstrated that all three enzymes are sensitive to H_2O_2 and that manganese is able to protect them. Surprisingly, we were also able to show that peptide deformylase and threonine dehydrogenase have highly reactive metal-coordinating cysteinyl residues.

3.2 RESULTS

3.2.1 PDF, TDH and CDA are sensitive to H_2O_2 *in vitro*

Peptide deformylase (PDF), threonine dehydrogenase (TDH) and cytosine deaminase (CDA) are mononuclear enzymes known to use multiple transition metals for activity *in vitro*. Since iron is the predominant metal in *E. coli*, we wondered if these mononuclear enzymes were metallated by iron *in vivo*. Because ferrous iron is readily oxidized to the ferric form by H_2O_2, sensitivity of these mononuclear enzymes to H_2O_2 *in vitro* would suggest that these enzymes use iron *in vivo*. We tested the sensitivity of these enzymes towards H_2O_2 in catalase mutants (*katE katG*) *in vitro*. KatE KatG mutants cannot scavenge H_2O_2 *in vitro* but are not H_2O_2-stressed *in vivo* due to the scavenging ability of AhpCF. We found that a brief challenge with low amounts of H_2O_2 (10 μM, 5 minutes) *in vitro* resulted in over 90% loss of PDF, 75% loss of TDH and 90% loss of CDA activity (Figure 1A). This result is consistent with the idea that mononuclear enzymes are iron-charged inside cells.

To further evaluate the sensitivity of these enzymes towards H_2O_2, we purified PDF and TDH. We wanted to confirm that iron-metallated PDF and TDH are indeed sensitive to H_2O_2. In contrast we expected these enzymes to be resistant to H_2O_2 if metallated with other transition metals, since unlike ferrous iron, other transition metals are not readily oxidized by H_2O_2. Both of these purified enzymes were thus metallated with various metals, including manganese and iron, and their sensitivity to low amount of H_2O_2 was measured. Iron-metallated variants of PDF and TDH were found to be extremely sensitive to H_2O_2, while manganese- (PDF, TDH), nickel- (PDF), cobalt- (PDF, TDH) and zinc- (PDF, TDH) metallated enzymes were resistant (Figure 1B, C). At 0° C, the H_2O_2 rate constants for inactivation of PDF and TDH ($\geq$ 2,200 and 7,200 M^{-1} s^{-1} respectively) were similar to those of Fe/S dehydratases [14] and Rpe [27] by H_2O_2. These high inactivation rates are similar to those of the Fenton reaction [19, 23], which imply that PDF and TDH are susceptible to Fenton-mediated damage *in vivo* by H_2O_2. While we were able to determine that the rate constant for inactivation is first order for TDH, due to experimental constraints we

were unable to test if that was the case for PDF as well. It is more than likely that the rate of inactivation of PDF is significantly higher than 2,200 $M^{-1} s^{-1}$ at 0° C.

3.2.2 PDF, TDH and CDA are also sensitive to H_2O_2 *in vivo*

Although our *in vitro* results are consistent with the idea that PDF, TDH and CDA are metallated with iron *in vivo*, we wanted to confirm this result. We tested this by challenging KatE KatG mutants with H_2O_2 *in vivo*. Our *in vitro* results with purified PDF and TDH suggest that metals bound to mononuclear enzymes, specifically iron and manganese, have high rates of dissociation from these enzymes (Table 3.2). This makes it likely that these enzymes are predominantly either mismetallated or in the apo form in crude extracts. In order to ensure that activities of enzymes in crude extracts represented fully metallated enzymes with a consistent metal, assays were anaerobically done in the presence of either nickel (PDF) or iron (PDF, TDH). As can be seen in Figure 1A, KatE KatG mutants challenged with a bolus of H_2O_2 *in vivo* (100 μM, 10 mins) have over 70% loss of PDF, TDH and CDA activity. Therefore PDF, TDH and CDA are metallated with iron *in vivo* and are thus vulnerable to damage by H_2O_2.

E. coli strains deficient in H_2O_2 scavengers accumulate up to 1 μM H_2O_2 when grown aerobically. This low level of protracted H_2O_2 stress is sufficient to activate the OxyR stress response, which makes it likely that cells are exposed to such low, yet toxic, levels of H_2O_2 in their habitat [19]. If PDF, TDH and CDA are indeed inactivated in a Fenton-mediated manner, these enzymes should be sensitive to such low levels of protracted H_2O_2 exposure *in vivo*. To test this, we assayed these enzymes in peroxide scavenging deficient (Hpx^-, *katE katG ahpCF*) cells. We observed that PDF, TDH and CDA activities were significantly lower in H_2O_2-stressed (Hpx^-) cells as compared to wild-type cells *in vivo* (Figure 2).

We have previously reported that manganese import is critical during H_2O_2 stress and showed that manganese does not scavenge H_2O_2 [1], and hypothesized that manganese import is required most likely to functionally replace iron in

metalloenzymes, and thereby protect these enzymes from Fenton-mediated damage. We showed that the inability of cells to import manganese in Hpx^- *mntH* mutants caused these cells to quickly stop growing. Manganese supplementation is able to restore growth in these mutants. Consequently we also previously showed that H_2O_2 stressed cells that are overloaded with iron, in Hpx^- *dps* mutants, also quickly stop growing due to lack of insufficient manganese [1, 19]. Cells require Dps during H_2O_2 stress to chelate excess iron and thereby minimize Fenton-mediated damage inside cells. Thus lack of Dps in H_2O_2-stressed cells increases the total amount of 'free' or chelatable iron, which also has the effect of minimizing the amount of manganese in these mutant strains – by decreasing the probability of manganese, versus iron, binding to an enzyme. We showed that small amounts of manganese supplementation in growth medium are able to rescue Hpx^- *dps* cells grown aerobically [1]. We therefore hypothesized that PDF, TDH and CDA activities should be lower in Hpx^- *mntH* and Hpx^- *dps* cells, as compared to Hpx^- cells, if manganese import is indeed required to functionally replace iron in these enzymes. As can be seen in Figure 2, PDF, TDH and CDA activities are extremely low in Hpx^- *mntH* and Hpx^- *dps* cells. As expected, manganese supplementation was able to restore wild-type levels of PDF, TDH and CDA activities (Figure 2). These results are consistent with the idea that manganese import is necessary, in H_2O_2-stressed cells, to functionally replace iron in mononuclear enzymes.

3.2.3 PDF overexpression rescues Hpx^- *mntH* and Hpx^- *dps* cells

Considering PDF is an essential enzyme, we wondered if the extremely low PDF activity in Hpx^- *mntH* and Hpx^- *dps* cells was the reason why these cells fail to grow when cultured aerobically. We overexpressed PDF, behind it own promoter in pBR322 and grew cells in defined medium (Casamino Acids and glucose). Over expression of PDF was able to relieve the Hpx^- *mntH* growth defect fully, and in part allowed Hpx^- *dps* to grow (Figure 3). While assays showed PDF activities were much higher in cells with the over expression plasmid, TDH and CDA activities were still low in these mutants (data not shown). This tells us that the extremely low

mononuclear enzyme activities in Hpx^- *mntH* and Hpx^- *dps* cells do not result from the growth defects of these mutants but rather is due to damage to these enzymes. Thus, during H_2O_2 stress, the growth defects caused by the inability of cells to import manganese, or to control the levels of 'free' chelatable iron, is due to damage to PDF.

3.2.4 Manganese protects mononuclear enzymes by functionally replacing iron in the active site

Since manganese import is required to keep mononuclear enzymes active during H_2O_2 stress, we wanted tested if PDF, TDH and CDA activities can be restored by manganese supplementation. As we have seen previously, manganese supplementation is not only able to relieve Hpx^- *mntH* and Hpx^- *dps* growth defects but also improves growth rates for Hpx^- cells [1]. Thus if the growth defects are linked to damage to these enzymes, manganese supplementation should be able to restore mononuclear enzyme activities. We found that just 5 μM $MnCl_2$ in growth medium (Casamino Acids + glucose) restore Hpx^- and Hpx^- *dps* PDF, TDH and CDA activities to near wild-type levels. Since Hpx^- *mntH* cells cannot actively import manganese, these mutants required 50 μM $MnCl_2$ in the growth medium to protect these enzymes.

We also tested the sensitivity of these enzymes to a large bolus of H_2O_2 (100 μM) for a short period of time (10 minutes) *in vivo* (Figure 4). We did this for two reasons: the large bolus of H_2O_2 for a short period of time makes it unlikely that this large amount of H_2O_2 would be quickly scavenged – by manganese for instance, and prevents any added protein induction from taking place. We also incubated the cultures with chloramphenicol, before H_2O_2 exposure, to ensure inhibition of protein synthesis. While enzyme activities in catalase (*katE katG*) mutants were severely reduced, PDF, TDH and CDA activities were protected in iron import mutants and Hpx^- cells. Although the total level of enzyme activities is lower in Hpx^- cells, the remaining activities are resistant to H_2O_2 stress. This sensitivity to H_2O_2 is most likely because of insufficient manganese in the medium, which prevents manganese

from fully metallating these enzymes. In the case of iron-import mutants, the lower levels of iron demetallates Fur protein, allowing the derepression of *mntH*. The induction of manganese transport and the lower levels of iron inside these cells allow manganese to metallate mononuclear enzymes such as PDF, TDH and CDA. These manganese metallated enzymes are predominantly resistant to H_2O_2 stress. However, even in iron-import mutants *mntH* is not fully depressed necessitating the supplementation of medium with additional manganese (10 μM, Figure 4). As mentioned before, in these experiments the genes induced by the OxyR stress response are not induced even during exposure to H_2O_2, due to the presence of chloramphenicol. Therefore, manganese is able to protect mononuclear enzymes against H_2O_2 *in vivo*, even in the absence of the protective effects of the OxyR response, most likely by functionally replacing iron.

Even though our results strongly support that manganese protects mononuclear enzymes from H_2O_2 by functionally replacing iron, we have been unable to directly show that these enzymes are metallated with manganese *in vivo*: cells with enzyme activities that are H_2O_2-resistant *in vivo* are not resistant to H_2O_2 *in vitro* in crude extracts. We have tried a range of different conditions but we have been unable to see H_2O_2 resistant activities in crude extracts in cells with *mntH* induced and fed with manganese (data not shown). In these experiments, in order to minimize the possible loss of manganese from mononuclear enzymes, we attempted to minimize the total time it would take to make extracts after harvesting cells. However, we found that enzyme activities from these extracts were still sensitive to H_2O_2, as if these enzymes were metallated by iron. Considering the high dissociation rate of manganese from PDF and TDH (Table 3.2) and the high concentration of iron in these cells, we reasoned that metal exchange in these enzymes occurs at too high of a rate to find manganese-bound and thus H_2O_2-resistant PDF, TDH and CDA in crude extracts. In order to test this idea, we incubated pure cobalt-charged PDF with concentrated extracts. Cobalt-charged PDF has a 100-fold lower dissociation rate as compared to manganese. If metal exchange is indeed really fast *in vivo*, then perhaps by incubating cobalt-metallated pure PDF with concentrated extracts we might be able to see cobalt exchanged for iron in PDF, which would lead to H_2O_2-sensitive

PDF. However, in this experiment we saw H_2O_2-resistant Co-PDF, thus no metal exchange had taken place (data not shown).

3.2.5 Cobalt supplementation also rescues Hpx$^-$ *mntH* and Hpx$^-$ *dps* cells, and protects against protein carbonylation

Since mononuclear enzymes can use multiple transition metals for activity, we wondered if a metal like cobalt, which *E. coli* does not naturally use, would also be able to protect cells in a manner analogous to manganese. Cobalt dismutes H_2O_2 at an extremely slow rate: in conditions ideal for dismutation of H_2O_2 by cobalt, the half-life of dismutation is over 200 minutes [7]. Thus if cobalt supplementation is able to help H_2O_2-stressed cells in a manner analogous to manganese then that would strongly suggest that manganese import is not required to scavenge H_2O_2. Cobalt supplementation was able to alleviate the growth defects of Hpx$^-$ *mntH* and Hpx$^-$ *dps* (Figure 5A) cells, and PDF and TDH activities were significantly higher in these cultures (data not shown). We have previously used protein carbonylation to measure the extent of protein damage in H_2O_2-stressed cells. Hpx$^-$ *dps* cells suffer high levels of protein carbonylation when cultured aerobically, which suggests that cells have many iron-charged mononuclear enzymes [1]. We have previously shown that manganese supplementation is able to alleviate protein carbonylation in Hpx$^-$ *dps* mutants [1] so we wondered if cobalt supplementation would also lead to the same result. Cobalt supplementation also prevented protein carbonylation in Hpx$^-$ *dps* cells (Figure 5B). These results further suggest that the necessity of H_2O_2-stressed cells to import manganese is not to scavenge H_2O_2, but to functionally replace iron in mononuclear enzymes.

3.2.6 Apo-PDF and apo-TDH are sensitive to H_2O_2

In our *in vitro* experiments with pure PDF and TDH, we observed that manganese-charged PDF and TDH were only resistant to H_2O_2 in the presence of

substrate (Figure 1 B, C). However, nickel-charged PDF and zinc-charged TDH, both metals with low dissociation rates from these enzymes (Table 3.2), were resistant to H_2O_2 even in the absence of substrate. Considering the high dissociation rate of manganese from these enzymes (Table 3.2), we wondered if there was a H_2O_2 sensitive residue in the active site of these enzymes. Both PDF and TDH use an active site cysteinyl residue to coordinate metal [5, 9]. Although H_2O_2 can directly oxidize cysteine, the oxidation rate of H_2O_2 with free cysteine is only 2 M^{-1} s^{-1} at neutral pH [32], which is not fast enough to be physiologically important. However, our initial estimates suggested a rate of apo-PDF and apo-TDH oxidation at least an order of magnitude higher than that. Therefore, we tested the ability of H_2O_2 to oxidize apo-PDF and apo-TDH anaerobically. We found that the oxidation of apo-PDF and apo-TDH happened at a very high rate of ~13000 M^{-1} s^{-1} for PDF and 1100 M^{-1} s^{-1} for TDH at room temperature. We were also able to show that oxidation of apo-PDF and apo-TDH is reversible: addition of TCEP, a reductant, is able to restore activity of the enzymes (Figure 6A, B). Similar results were also seen with another reductant, DTT. Since the oxidized enzyme is repairable with a reductant, it is likely that the cysteinyl residue has been oxidized either to the sulfenic acid or sulfenyl-amide form.

We wanted to directly show that it was indeed the active site cysteinyl residue that was vulnerable to H_2O_2 damage in PDF and TDH. Iodoacetamide inactivates iron charged PDF and TDH by reacting with the active site cysteine, presumably preventing metal association in the active site (Fig 7A). This shows that the cysteinyl residue is critical for enzyme activity. Using monobromobimane, a compound that only binds to sulfhydryls and fluoresces in the process, we were able to show that low amounts of H_2O_2 were able to prevent monobromobimane association with PDF (Fig 7B). This result shows that H_2O_2 is directly damaging the cysteinyl residue. Concurrently we were able to show that nickel charged PDF does not bind monobromobimane: the tight binding of nickel precludes the cysteine from reacting with the compound. Due to technical issues with the experiment, we were unable to show that oxidation of iron-metallated PDF also prevents monobromobimane binding. Based on our results with the apo enzyme, we would consider this likely.

Based on our results, we wondered if cysteine oxidation of PDF and TDH is physiologically important. With a half-life of inactivation around 10 min at 1 μM H_2O_2 it seems likely that the active site residue of PDF and TDH are prone to oxidation *in vivo* at physiologically relevant concentrations of H_2O_2. However we were unable to restore PDF and TDH activities of Hpx^- cells, to wild-type levels, by adding TCEP in cellular extracts (data not shown). We reasoned that sulfhydryl oxidation *in vivo* could lead to two types of cysteinyl residues: 1) reversibly oxidized forms i.e. sulfenic acids and sulfenyl-amides, and 2) irreversibly, over-oxidized forms i.e. sulfinic and sulfonic acid forms. It seems likely that the irreversible loss in PDF and TDH activity we see in H_2O_2-stressed strains *in vivo* is due over-oxidation of these enzymes. This implies that the reversibly oxidized forms of these enzymes are repaired *in vivo*. This repair rate appears to be quite fast: even if we quickly harvested and sonicated cells, we were unable to observe reversibly oxidized forms of these enzymes when cells were treated with H_2O_2 *in vivo* (data not shown). We also checked to see if PDF and TDH could be irreversibly oxidized *in vitro*. If so, then such a result could help explain the oxidation state of PDF and TDH *in vivo*. We treated pure apo-PDF and apo-TDH with a large bolus of H_2O_2 (100 μM) for 1 minute. We did this in the presence or absence of TCEP, to check to see if the presence of a reductant prevents over-oxidation of the apo enzyme. Our results show that apo-PDF and apo-TDH can be over-oxidized by H_2O_2, and the presence of TCEP prevents over-oxidation. As expected, these over-oxidized enzymes are non-repairable by a reductant (Figure 8A, B).

3.2.7 Preferential oxidation of active site cysteine protects further damage to PDF

We wondered why enzymes would use active site cysteinyl residues with such a high oxidation rate. Perhaps these residues are used so that iron-charged mononuclear enzymes preferentially get oxidized at such residues, sparing the polypeptide from irreversible covalent damage mediated through the Fenton reaction. We tested this idea by inactivating apo or iron-charged PDF with a short bolus of

H_2O_2, sufficient to completely inactivate iron-charged PDF, but not apo-PDF. H_2O_2 inactivation of iron-charged PDF could happen in two ways: 1) H_2O_2 oxidizes ferrous iron to the ferric form, after which the produced ferryl radical reacts with a random protein residue or 2) H_2O_2 oxidation of iron-charged PDF leads to the simultaneous oxidation of the metal coordinating cysteinyl residue. If the inactivation mechanism happens in accordance with the first possibility, we would expect to see a certain fraction of the oxidized iron-charged PDF in the reduced apo form. If the latter possibility occurs, then we would expect the metal coordinating cysteinyl residue to be completely oxidized. We were unable to observe PDF in the apo form when iron-charged PDF was challenged with H_2O_2 (Figure 9A, B). This result suggests that the cysteinyl residue is preferentially oxidized to protect the rest of the polypeptide. Consistent with this idea, we were unable to observe reversibly oxidized PDF challenged with H_2O_2 *in vivo* (data not shown). The only damaged population of PDF we observe is in the irreversibly damaged state. We also did not observe carbonylation of PDF in the Hpx^- background with PDF overexpressed (Figure 10A). This suggests that other peptide residues in PDF do not get damaged. It is important to note that the polypeptide is not degraded *in vivo* in these situations (Figure 10B).

3.2.8 Repeated rounds of damage can irreversibly inactivate PDF and TDH

PDF in cells exposed to H_2O_2 is irreversibly oxidized, especially in the absence of manganese import (Figures 3, 4). While we have been able to irreversibly damage PDF (Figure 8A, B) *in vitro* by over-oxidizing the active site cysteinyl residue, the over-oxidation of PDF is prevented if the same experiment is performed in the presence of a reductant, TCEP. Considering the reducing environment of the cell, we wanted to demonstrate that irreversible oxidation of PDF can also occur *in vitro* even in the presence of TCEP, to ensure that our *in vivo* and *in vitro* results were in agreement. In order to do this we incubated iron-charged PDF aerobically in the presence of TCEP, and used nickel-charged PDF as our control. As can be seen in Figure 11, PDF slowly gets irreversibly damaged. Thus while the active site cysteinyl residue limits the damage sustained to the polypeptide, repeated cycles of

inactivation of iron-charged PDF with H_2O_2 eventually results in an irreversibly-oxidized enzyme *in vitro* (Figure 11). Two possibilities exist: either cysteinyl residue gets irreversibly oxidized, and/or the oxidation of other residues on the protein result in an inactive protein. Considering we do not see carbonylated PDF *in vivo*, and that we can over-oxidize the active site cysteinyl residue *in vitro*, the former possibility is more likely than the latter. Perhaps the oxidation of the cysteinyl residue from the reversible to the irreversible oxidized state is sufficiently fast that over time enough of the irreversible oxidized state accumulates.

3.3 DISCUSSION

3.3.1 Mononuclear enzymes are sensitive to H_2O_2

We have previously shown that H_2O_2-stressed cells, which are lack *dps* and therefore cannot chelate excess iron, exhibit extremely high levels of protein carbonylation – which is indicative of hydroxyl-radical formation [1]. Since manganese supplementation is able to prevent protein carbonylation and does not do so by scavenging H_2O_2, we reasoned that 1) the iron-loaded enzymes must be damaged by a Fenton reaction, and 2) that manganese must be protecting mononuclear enzymes – normally metallated with iron – from oxidative damage, by occupying the metal site. In a recently published work, RPE was shown to be a mononuclear enzyme that is sensitive to H_2O_2 stress *in vivo* [27]. In this work we have shown that three additional and yet otherwise unrelated enzymes use iron as a mononuclear cofactor *in vivo* and that these enzymes are sensitive to physiologically relevant concentrations of H_2O_2. These three mononuclear enzymes, like RPE, can also use multiple transition metals for activity. According to current literature there are over one hundred mononuclear enzymes in *E. coli* (www.ecocyc.org). Iron is frequently overlooked as a possible cofactor for these divalent cation-utilizing enzymes because ferrous iron is quickly oxidized to the insoluble ferric form in the presence of oxygen. In the reducing environment inside cells, iron is kept mostly in the ferrous form. Since ferrous iron is the predominant metal *in vivo* in *E. coli* [18], it

is likely that many such mononuclear enzymes are metallated with iron as opposed to other transition metals *in vivo*. Presumably all such iron-metallated mononuclear enzymes are susceptible to damage by physiologically relevant concentrations of H_2O_2.

If iron-charged mononuclear enzymes are so sensitive to H_2O_2 stress, why does the cell continue to use iron as a cofactor for such enzymes? The role of metals in mononuclear enzymes is to provide a local positive charge, serve as an electron sink and help in substrate binding. With a coordination number of up to six, transition metals provides enzymes with tremendous ability to bind ligands and facilitate reactions. In contrast, divalent cations, like magnesium, are not geometrically flexible in binding multiple ligands and thus are not used in such enzymes where the cation, i.e. metal, goes through several geometrical conformations during the course of a reaction. So while multiple transition metals can be used in mononuclear enzymes, *E. coli* depends on using iron, and in certain situations manganese, as opposed to other transition metals. This might be for two main reasons: 1) availability of iron is higher in the natural habitat of *E. coli* and 2) manganese and iron seem to be the only cofactors universally active with these enzymes. For instance, PDF is highly active with nickel, but nickel inhibits TDH activity. Moreover, zinc-metallated TDH has fairly high activity but zinc-metallated PDF activity is extremely low. The choice of iron in such enzymes might also be evolutionary: iron was abundant and readily available in the ferrous form when oxygen had not permeated in the earth's atmosphere. Thus, the dependence of cells on iron might be a requirement that they inherited from ancient organisms.

It would be interesting to test the metallation status of such enzymes in obligate aerobes such as *Pseudomonas aeruginosa* or in organisms that do not depend on iron for metabolism, but rather manganese, such as *Lactobacillus plantarum* and *Borrelia burgdorferi*. Are mononuclear enzymes in these organisms metallated with manganese? If so, does expression of these enzymes in an organism like *E. coli* allow them to be functional with iron? If true, that would suggest that metallation of such

enzymes is indeed dependent on their environment as opposed to the inherent affinity of the enzyme.

3.3.2 Enzyme active site sulfhydryls are targets of H_2O_2

Thiols were once suspected of being primary reactive oxygen species (ROS) targets. Support for this idea came from the induction of thioredoxin and glutaredoxin by the H_2O_2 sensor of the cell, OxyR. However, it has proven hard to find sulfhydryls that are damaged by H_2O_2 at physiologically relevant rates. For the first time we have shown two enzymes with sulfhydryls that are extremely susceptible to H_2O_2 stress at physiologically relevant concentrations of H_2O_2.

The rates of oxidation of sulfhydryls in biology vary by orders of magnitude: While sulfhydryls on H_2O_2 sensors such as OxyR are oxidized at rates in excess of 1×10^5 M^{-1} s^{-1} [3], oxidation rates of sulfhydryls on other proteins have not been reported to be higher than 60 M^{-1} s^{-1}. For instance, oxidation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by H_2O_2 occurs at a rate of 40-60 M^{-1} s^{-1} [11, 33]. The oxidation of protein tyrosine phosphatases by H_2O_2, thought to play a role in regulating enzyme activity, occurs at a rate of 10-20 M^{-1} s^{-1}. Other thiol containing enzymes such as carbonic anhydrase III [17], isocitrate lyase ([12, 22], data not shown) and α1-antitrypsin [10] require several minutes of millimolar levels of H_2O_2 exposure to completely oxidize the sulfhydryls on these proteins. In contrast, the rate of oxidation of active site sulfhydryls in PDF and TDH occur at a high rate of 1×10^4 and 1×10^3 M^{-1} s^{-1} respectively. At 1 μM H_2O_2 the half-life for PDF and TDH oxidation is only ~ 1 and ~10 min respectively as compared to ~ 10 h for isocitrate lyase and ~3 h for GAPDH. Thus cysteinyl oxidation in PDF and TDH *in vivo* is a significant problem for cells, unlike oxidation of isocitrate lyase and GAPDH.

Our results are consistent with the idea that the preferential oxidation of the active site sulfhydryl protects PDF and TDH from irreversible protein damage. Thus the cysteinyl residue is sacrificial to prevent irreversible Fenton-mediated damage to other peptidyl residues of the enzyme. This could also explain why the cysteinyl

residue is so reactive in PDF and TDH. However, why do these enzymes have such a reactive sulfhydryl as opposed to other enzymes with active site cysteines such as isocitrate lyase? This might be because the enzyme requires a strongly nucleophilic residue to bind iron and manganese. Interestingly, other mononuclear enzymes such as RPE [16] and CDA [13] do not require an active site cysteine for activity. While RPE has recently been shown to be irreversibly damaged due to repeated cycles of Fenton chemistry [27], we have shown that PDF also suffers the same fate. However, in the latter case, the active site cysteine might be preferentially over-oxidized to sulfinic and sulfonic acids. It is yet to be determined if sulfinic and sulfonic residues are possibly repairable *in vivo*.

There is considerable uncertainty, and variance in the products of sulfhydryl oxidation by reactive oxygen species. Sulfhydryl oxidation on enzymes have been shown to lead to sulfenic, sulfinic, sulfonic, sulfenyl-amide and disulfide bonds. Of these states, only sulfenic and sulfenyl-amide have been shown to be reversible [8, 20, 24, 30]. The significance of these states in biology is not yet fully understood but a range of ideas exist: while sulfenic acids are thought to be involved in regulating enzyme function, the formation of sulfenyl-amides are thought to prevent over oxidation of sulfhydryl residues. The product(s) PDF and TDH cysteinyl oxidation is also not clear. We think that the active site sulfhydryl residues of PDF and TDH are quickly oxidized to the sulfenic form, which can then be over-oxidized to the sulfinic and sulfonic form. However, we have not observed the presence of sulfenic or sulfinic acid on PDF. While we have been unable to perform mass spectrometry on TDH, the low mass of PDF makes it unlikely to determine the presence of a sulfenyl-amide by ESI-MS. We have only been able to observe a mass increase of 48, which would correspond to a sulfonic acid (data not shown). X-ray crystallography might be able to answer if oxidation of PDF does indeed lead to the formation of sulfenyl-amide.

The high oxidation rate of active site sulfhydryls on PDF and TDH demonstrate that oxidation of some cysteines *in vivo* occurs at physiologically

relevant rates. Why these enzymes have such reactive sulfhydryls warrants further investigation.

3.3.3 Role of manganese in mononuclear enzymes

Regulation of manganese transport in *E. coli* suggests that manganese import is important during both iron starvation and H_2O_2 stress. During iron starvation, derepression of *mntH* due to deactivation of Fur allows the cells to import manganese and most likely metallate enzymes. As we have shown in this work, mononuclear enzymes in iron-starved cells are resistant to a H_2O_2 bolus even in the absence of activation of OxyR stress response. This result is consistent with the idea that manganese is able to functionally replace iron in mononuclear enzymes. In H_2O_2-stressed cells, *mntH* is critical in protecting mononuclear enzymes from damage, as can be seen in the growth defect of Hpx$^-$ *mntH* cells – due to damage to PDF. Even in iron overload conditions, such as in Hpx$^-$ *dps*, small amounts of manganese supplementation are able to protect mononuclear enzymes from Fenton-mediated damage.

As of yet, we have been unable to directly show that mononuclear enzymes are metallated with manganese: in conditions that mononuclear enzymes are resistant to H_2O_2 *in vivo* and thus should be metallated with manganese, in cell extracts these enzymes are sensitive to H_2O_2 *in vitro* and appear to be metallated with iron. Considering the high dissociation rate of manganese from these enzymes this result is not surprising. Although other researchers have proposed that manganese must be acting as a ROS scavenger, we have shown previously that this is at least not true for *E. coli* [1]. *Lactobacillus plantarum* continues to produce millimolar levels of H_2O_2 even though the organism is replete with manganese [2]. Further, like manganese, cobalt supplementation is also able to relieve both Hpx$^-$ *mntH* and Hpx$^-$ *dps* growth defects, prevent protein carbonylation in these mutants and protects CDA [21], PDF and TDH against H_2O_2 *in vitro*. Due to the much higher reduction potential of cobalt as compared to manganese, it is unlikely that cobalt acts as a ROS scavenger *in vivo*.

Thus relief of these growth defects by cobalt further suggests that manganese must be functionally replacing iron *in vivo*.

The high dissociation rates of manganese and iron from RPE [27], PDF and TDH make it unlikely that we can directly observe manganese binding to these enzymes *in vivo*. However as mentioned before, these high dissociation rates likely allow cells to quickly regulate metabolism based on metal availability. Other metals, such as zinc, cobalt and nickel, which are also resistant to H_2O_2 but have high binding affinities to mononuclear enzymes might not be used – to prevent poisoning of other enzymes. To prevent incorrect metallation, the cell may actively prevents metals like zinc from binding these enzymes. It is not clear if such a mechanism exists, but it seems plausible. Certain proteins have been shown to only change conformation when bound to their cognate metal, as opposed to zinc [28]. The metallation status of metalloenzymes in other organisms, such as *Salmonella*, which requires cobalt import, could help explain the existence of such a system, since cobalt not only binds tightly to PDF and TDH but also has higher catalytic efficiency as compared to zinc. Thus if *Salmonella* continues to use iron and/or manganese to metallate its mononuclear enzymes then that could indicate if the cell actively removes cobalt from the active site of such enzymes. However, it is more likely that cobalt import is strictly controlled inside the cell, such as nickel in *E. coli*, and thus cobalt is never allowed to interact with mononuclear enzymes.

3.4 EXPERIMENTAL PROCEDURES

3.4.1 Reagents

Acid-hydrolyzed casamino acids (Hy-Case Amino), amino acids, ascorbic acid, antibiotics, o-nitrophenyl-β-D-galactopyranoside (ONPG), catalase (from bovine liver), ferrous ammonium sulfate hexahydrate, cytosine, cobalt (II) chloride hexahydrate, manganese (II) chloride tetrahydrate, nickel sulfate, zinc chloride, diethylenetriaminepenta-acetic acid (DTPA), EDTA, NADH, NAD, monobromobimane, D, L-dithiothreitol (DTT) and TCEP were from Sigma-Aldrich. Guanidine hydrochloride was obtained from Fisher Scientific. Formate dehydrogenase (*Candida boidinii*) was from Roche. Formyl-Met–Ala–Ser was from Bachem. OxyBlot Protein Oxidation Detection Kit (S7150) was from Chemical International.

3.4.2 Bacterial growth

Luria broth (LB) contained (per liter) 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl. Minimal A casein hydrolysate/glucose medium (MinCAAG) consisted of minimal A salts (Miller 1972) supplemented with 0.2% casein hydrolysate, 0.5 mM L-tryptophan, and 0.2% glucose. When antibiotic selection was needed, media were supplemented with either 100 μg/mL ampicillin or 20 μg/mL chloramphenicol. Protein synthesis was stopped by adding 150 μg/mL chloramphenicol.

Anaerobic cultures were grown in an anaerobic chamber (Coy Laboratory Products Inc.) under an atmosphere of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. Aerobic cultures were grown with vigorous shaking at 37°C. To ensure that cells were growing exponentially before they were exposed to oxygen, anaerobic overnight cultures of oxygen-sensitive strains were diluted to OD_{600} = 0.005 in fresh anaerobic medium and allowed to grow to OD_{600} ~ 0.15 at 37°C. These cells were

then subcultured into fresh aerobic medium to obtain an OD_{600} of 0.005, with or without manganese chloride, and grown aerobically at 37°C.

3.4.3 Strains and strain construction

Strains used in this study are listed in Table 3.1. All constructions in Hpx^- (i.e. *katE katG ahp*) backgrounds were performed in an anaerobic chamber to ensure that suppressor mutations were not selected during outgrowth. Null mutations were created using the Red recombinase method (Datsenko and Wanner, 2000). Mutations were introduced into new strains by P1 transduction (Miller, 1972). The resultant mutations were confirmed by PCR analysis and, when possible, by enzyme assays.

3.4.4 Plasmid construction

The plasmid pPDF expressing the wild-type *def* allele and its promoter region was made using the forward primer 5'-
GCAATGGAATTCTTCTGTATCGACCATCCTTAT
CTCCCTG and reverse primer 5'- with
GCAATGGGATCCTTAAGCCCGGGCTTTCA
GACGATC with EcoRI and BamHI restriction sites, respectively. The PCR product was inserted into pBR322 and confirmed by sequencing. The plasmid pPDF-FLAG expressing the wild-type *def* allele tagged with a FLAG tag on the C terminus was made using the forward primer 5'-
GCAATGGAATTCGAACACATCTCTGGAGATTTATG TCAGTTTTGCAAG and reverse primer 5'-GCAATGTCTAGATTATTTATCATCATC
ATCTTTATAATCAGCCCGGGCTTTCAGACG with EcoRI and XbaI restriction sites, respectively. The PCR product was inserted into pWKS30 [31] and confirmed by sequencing. The plasmid pPDF-PET21B, for over-expressing PDF, was made using the forward primer 5'-
GCAATGCATATGTCAGTTTTGCAAGTGTTACATATTCCG and reverse primer

5’- GCAATGCTCGAGTTAAGCCCGGGCTTTCAGACGATC with NdeI and XhoI restriction sites, respectively. The PCR product was inserted into pET21B and confirmed by sequencing. The plasmid pTDH-PET21B, for over-expressing TDH, was made using the forward primer 5’-GCAATGCATATGAAAGCGT TATCCAAACTGAAAGCG and reverse primer 5’-GCAATGGGATCCTTAATCCCA GCTCAGAATAACTTTCCC with NdeI and BamHI restriction sites, respectively. The PCR product was inserted into pET21B and confirmed by sequencing.

3.4.5 Protein purification

PDF and TDH were purified aerobically by FPLC. PDF was purified as cited [26], with slight modifications. A single colony of *E. coli* BL21 (DE3) containing pET21b-PDF was inoculated in 5 mL LB media with 100 μg/mL ampicillin and grown overnight at 37° C. This overnight culture was used to inoculate 500 mL LB media with 100 μg/mL ampicillin. The culture was grown at 37° C to OD (600 nm) of ~ 0.5, after which cells were induced with 0.05 mM IPTG and grown at 18° C for 20 hours, before harvesting. The harvested cells were washed in buffer A (50 mM HEPES (pH 7.5) with 10 mM NaCl and 0.2 mM $NiSO_4$) and then resuspended in the same buffer. These cells were lysed in a French press and cell debris removed by centrifugation at 22,000 x *g* for 20 min. PDF protein was purified using three steps. The extract was first separated by ion exchange chromatography (GE HiTrap Q XL) with equilibration in buffer A and elution with a linear gradient of 10–500 mM NaCl. The fractions with the highest PDF activity were pooled, concentrated (Millipore Amicon Ultra 3K) and washed with buffer B (10 mM potassium phosphate buffer pH 6.8). The pooled concentrated fractions were then separated using hydroxyapatite chromatography (BioRad CHT type I, 40 μM) with elution using a linear gradient of 10–400 mM potassium phosphate. The fractions with the highest PDF activity were pooled, concentrated, and washed in buffer A. These pooled fractions were then separated using size exclusion chromatography (GE Hiload 16/60 Superdex 75) with

elution in buffer A. Fractions with highest PDF activity were pooled, concentrated and frozen with 40% glycerol in buffer A at stored at -80° C.

TDH was purified as cited [4], with modifications. A single colony of *E. coli* BL21 (DE3) containing pET21b-TDH was inoculated in 5 mL LB media with 100 μg/mL ampicillin and grown overnight at 37° C. This overnight culture was used to inoculate 500 mL LB media with 100 μg/mL ampicillin. The culture was grown at 37° C to OD (600 nm) of ~ 0.5, after which cells were induced with 0.05 mM IPTG and grown at 18° C for 20 hours before, harvesting. The harvested cells were washed in buffer C (50 mM Tris (pH 8.4) with 100 mM KCl and 0.25 mM $ZnCl_2$) and then resuspended in the same buffer. These cells were lysed in a French press and cell debris removed by centrifugation at 22,000 x *g* for 20 min. The extract was first separated by ion exchange chromatography (GE HiTrap Q XL) with equilibration in buffer C and elution with a linear gradient of 100–1000 mM KCl. The fractions with the highest TDH activity were pooled, concentrated (Millipore Amicon Ultra 3K) and washed with buffer C (50 mM HEPES buffer pH 7.5 with 100 mM KCl and 0.2 mM $ZnCl_2$). The pooled fractions were then applied to a blue dextran-Sepharose column (GE HiTrap Blue SP) with elution using a pulse of 50 mL 20 mM NAD^+. During elution, protein was tracked by monitoring absorbance at 475 nm wavelength. Fractions with highest TDH activity were pooled, concentrated and frozen with 40% glycerol in buffer C at stored at -80° C.

3.4.6 Enzyme assays

To assay enzymes, cells were washed twice and resuspended in anaerobic buffer. All enzyme assays were done anaerobically, unless otherwise stated. Extracts were lysed by sonication and cell debris removed anaerobically. All enzyme assays were performed at 25° C unless specified otherwise specified. Peptide deformylase (PDF) [15], threonine dehydrogenase (TDH) [4], and cytosine deaminase (CDA) [21] assays were measured according to cited methods, with slight modifications. Peptide deformylase was assayed in 50 mM HEPES buffer (pH 7.5) with 25 mM NaCl. A

typical assay consisted of enzyme (pure or extract) 10 mM NAD, 1 unit of formate dehydrogenase, 1 mM Formyl-Met–Ala–Ser and 100 μM of metal. Threonine dehydrogenase was assayed in 50 mM Tris-HCl buffer (pH 8.4) with enzyme (pure or extract), 1 mM NAD, 30 mM threonine and 100 μM of metal. PDF and TDH activities were measured at 340 nm. A typical assay of CDA included buffer, enzyme (extract) 100 μM metal and 400 μM cytosine. CDA activities were measured by monitoring cytosine disappearance at 286 nm. Galactosidase activity was measured by ONPG hydrolysis (Miller, 1972), and total protein content was determined using the Coomassie blue dye-binding assay (Pierce).

3.4.7 *In vitro* studies

Pure PDF was stored metallated with $NiSO_4$. To metallate PDF with other metals, pure PDF was incubated for ~45 min with 25 mM EDTA to chelate nickel. The chelated enzyme was then diluted 1:100 and metallated with various metals. Pure TDH was stored metallated with $ZnCl_2$. To metallate TDH with other metals, TDH was incubate for ~30 min with 10 mM EDTA to chelate zinc. The chelated enzyme was then diluted 1:50 and metallated with various metals.

PDF and TDH, metallated with various metals, were challenged with 10 μM H_2O_2 for 5 min with substrate present. Presence of substrate is required for manganese- and iron- charged enzymes or the enzymes lose the metals quickly. Substrate offers no protection against H_2O_2 since increasing substrate concentration has no effect on rate of inactivation (data not shown).

Rate of inactivation of apo-PDF by H_2O_2 was measured as follows: apo-PDF was incubated with H_2O_2 (1 μM) and at various time points an aliquot of enzyme was added to a tube containing catalase (1:1000 dilution), $NiSO_4$ (500 μM) and +/- TCEP (500 μM). The rate of apo-TDH inactivation was determined similarly, except $ZnCl_2$ (500 μM) was used instead of $NiSO_4$. Over oxidation of apo-PDF and apo-TDH were done similarly except 100 μM H_2O_2 was used for 1 min. Samples were incubated +/-

TCEP (500 μM) and samples without TCEP during H_2O_2 exposure were checked for over-oxidation by adding TCEP (500 μM) afterwards. All *in vitro* H_2O_2 were done anaerobically, unless stated otherwise. The samples were then assayed as described above.

To check if iodoacetamide can inactivate PDF and TDH, pure iron-metallated PDF and TDH were incubated with 25 mM iodoacetamide for 45 min at 37° C. These samples were then assayed as described above.

Fluorescence determination of monobromobimane binding was measured on a NanoDrop 3300 fluorospectrometer. Excitation wavelength was 380 nm and fluorescence was measured at 475 nm. Nickel-charged PDF and apo-PDF were challenged with 20 μM H_2O_2 for 1 min, after which the samples were incubated with 500 μM monobromobimane for 30 min in the dark. Fluorescence was then measured.

To check if the cysteinyl residue in PDF is preferentially oxidized by H_2O_2, an assay with pure iron-metallated PDF, in the presence of 100 μM ferrous ammonium sulfate, was started and incubated for 3 min. DTPA (200 μM) was added and incubated for 30 sec, followed by 50 μM H_2O_2 challenge for 5 sec, after which catalase (1:1000 dilution), $NiCl_2$ (500 μM) and TCEP (500 μM) were added as indicated. For apo-PDF controls, apo-PDF was incubated with DTPA (200 μM) and challenged with 50 μM H_2O_2 for 5 seconds; catalase (1:1000 dilution) was then added, followed by $NiCl_2$ (500 μM) and TCEP (500 μM) as indicated.

To measure the loss of ferric iron from the active site of PDF, an assay with iron-metallated PDF in the presence of 100 μM ferrous ammonium sulfate, was started and incubated for 3 min. DTPA (200 μM) was added and incubated for 30 sec followed by addition of $NiCl_2$ (500 μM) and TCEP (500 μM). Assay was then challenged with 25 μM H_2O_2 for 5 sec, after which catalase (1:1000 dilution) was added. The rate of TCEP reduction of apo-PDF was measured by challenging apo-PDF with 25 μM H_2O_2 for 20 sec, in the presence of DTPA (200 μM). Catalase (1:1000 dilution), TCEP (500 μM) and $NiCl_2$ (500 μM) were then added.

To look at the effect of ascorbate cycling on PDF, iron-charged and nickel-charged PDF with TCEP (500 μM) were incubated +/- O_2. At time intervals an aliquot of enzyme was moved to the anaerobic chamber and was then assayed for activity in the presence of 500 μM $NiCl_2$.

3.4.8 *In vivo* H_2O_2 exposure

To check the effect of *in vivo* H_2O_2 exposure, Hpx^- or *katE katG* mutants were used to prevent H_2O_2 scavenging by catalase. Although catalase mutants, *katE katG* mutants do not experience H_2O_2 stress when grown aerobically due to the presence of *ahpCF*. For *in vivo* exposure with a bolus of H_2O_2, cells were grown at 37° C +/- $MnCl_2$, as indicated, to OD (600 nm) of ~0.2–0.3. Protein synthesis was inhibited by adding 150 μg/mL chloramphenicol for 15 min. Cells were then stressed with 100 μM H_2O_2 for 10 min, followed addition of catalase (1:5000 dilution). Cells were then washed with 50 mM HEPES with 25 mM NaCl, resuspended in the same buffer and then sonicated. PDF, TDH and CDA were then immediately assayed as described.

3.4.9 Protein carbonylation

Cells were grown anaerobically in defined medium (MinCAAG) at 37° C to an OD (600 nm) of 0.1-0.15. Strains were diluted to an OD (600 nm) of 0.005 in freshly made aerobic defined medium (MinCAAG), +/- $MnCl_2$ or +/- $CoCl_2$. Hpx^- *dps* cells, grown without $MnCl_2$ or $CoCl_2$, were subcultured to an OD (600 nm) of 0.050, since these cells double in biomass only twice before growth ceases. Cells were harvested at an OD (600 nm) of ~ 0.2. These cells were washed twice with ice-cold 50 mM potassium phosphate buffer pH 7.0, after which they were suspended in 500 μL of the same buffer with the addition of 5 mM DETAPAC. DETAPAC prevents further protein oxidation in extracts. Cells were sonicated, and then protein carbonylation was measured using the OxyBlot Protein Oxidation Detection Kit (S7150) (Chemicon International). Protein carbonyl groups were derivatized to their

2,4-dinitrophenylhydrazones by reaction with 2,4-dinitrophenylhydrazine (DNPH) for 15 min in 3% (w/v) SDS. β-Mercaptoethanol (1% v/v) was then added to these derivatized samples, after which the samples were subjected to polyacrylamide denaturing gel electrophoresis (4-15% BioRad). Proteins were then transferred to a PVDF membrane (Amersham HybondTM-ECLTM by GE Healthcare) for 120 min at 20 V. The membrane was then incubated with primary antibody specific to the DNP moiety attached to the derivatized proteins. This step was followed by incubation with a horseradish peroxidase antibody conjugate directed against the primary antibody. The membranes were then treated with chemiluminescent substrate (GE Healthcare, Amersham ECLTM Western Blotting Analysis System) and imaged by exposure to light sensitive films (Amersham Hyperfilm ECLTM chemiluminescence film, GE Healthcare, Buckinghamshire, UK).

3.4.10 Tracking PDF polypeptide *in vivo*

MG1655, LC106 and AA30 containing pPDF-FLAG were grown anaerobically with 2.5 mM lactose to OD (600 nm) ~ 0.2. Chloramphenicol (150 μg/mL) was then added to inhibit protein synthesis. After 15 min cells were allowed to grow aerobically, and at specified time intervals samples were harvested. These cells were washed with 50 mM HEPES containing 25 mM NaCl and then resuspended in the same medium. The samples were then western blotted as described above, using ProteoQwest anti-FLAG peroxidase conjugate antibody.

3.5 TABLES

Table 3.1. Strains

Strain	Paper genotype	Reference
MG1655	F^-, wild type	E. *coli* Genetic Stock Center
LC106	*ΔahpCF' kan::'ahpF Δ(katG17::Tn10)1 Δ(katE12::Tn10)1*	Seaver and Imlay (2004)
AA30	As LC106 plus Δ*mntH2*::*cat*	Anjem *et al.* (2009)
JI367	*katE12::Tn10 Δ(katG17::Tn10)1*	Seaver and Imlay (2001)
GR538	*ΔzupT::cat ΔentC ΔfeoABC ΔfecABCDE*	Grass *et al.* (2005)
AA301	*ΔkatE1::kan katG17::Tn10 ΔzupT::cat ΔentC ΔfeoABC ΔfecABCDE*	This work
BL21 (DE3)	F^- *ompT gal dcm lon* $hsdS_B$ (r_B^- m_B^-) λ (DE3)	Novagen
Plasmids		
pPDF	pBR322::*def*	This work
pET21b-PDF	pET21b::*def*	This work
pET21b-TDH	pET21b::*tdh*	This work
pPDF-FLAG	pWKS30::*def*-FLAG	This work

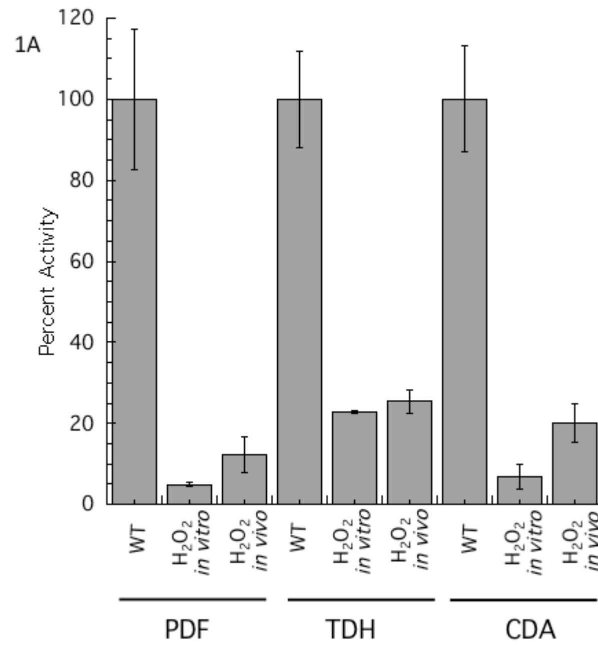
Table 3.2. Kinetic values of PDF and TDH

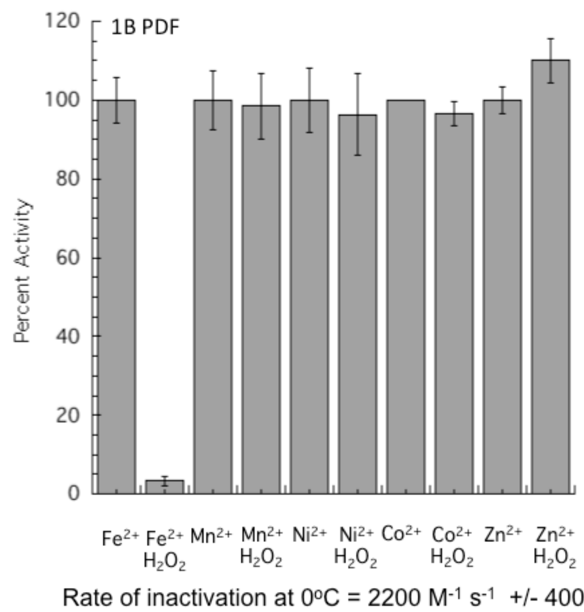
	k_{cat} (s^{-1})	K_M (mM)	k_{cat} /K_M (mM^{-1} s^{-1})	$t_{1/2}$ of metal dissociation
FePDF	64.0	5.3	12.0	6.6 min
NiPDF	69.0	5.9	11.5	60.0 min
CoPDF	32.0	7.4	4.3	120.0 min
MnPDF	15.0	6.9	2.3	1.5 min
ZnPDF	0.04	>9.0	<0.004	148.0 min

	k_{cat} (s^{-1}, Thr)	K_M (mM, NAD)	K_M (mM, Thr)	k_{cat} /K_M (mM^{-1} s^{-1}, Thr)	$t_{1/2}$ of metal dissociation
FeTDH	88.0	0.27	6.5	13.5	8.0 min
ZnTDH	18.0	ND	2.8	6.4	113.0 min
CoTDH	6.0	ND	0.97	5.8	133.0 min
MnTDH	18.0	0.27	123.0	0.15	13.0 min

3.6 FIGURES

Figure 3.1





Rate of inactivation at $0^{o}C$ = 2200 M^{-1} s^{-1} +/- 400

Figure 3.1 (Continued)

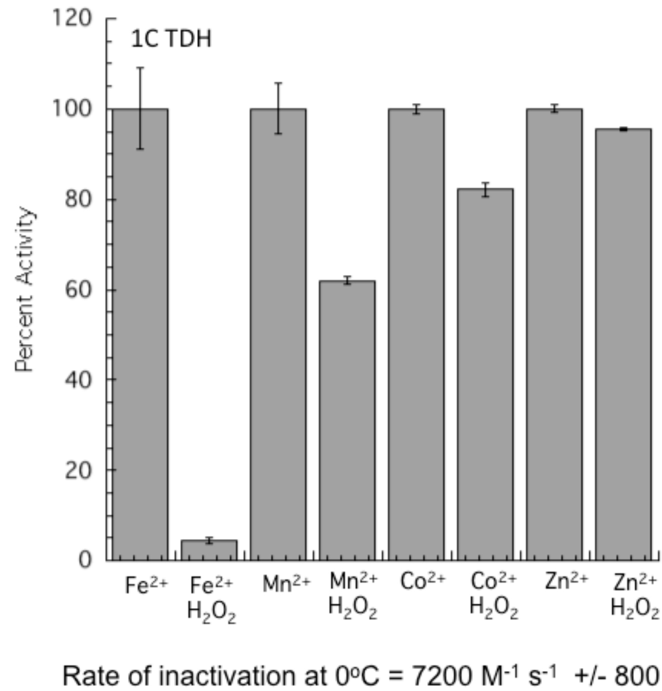
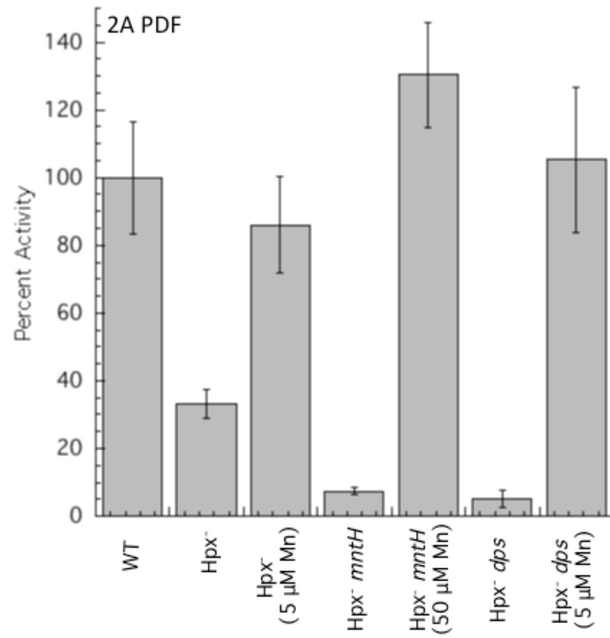
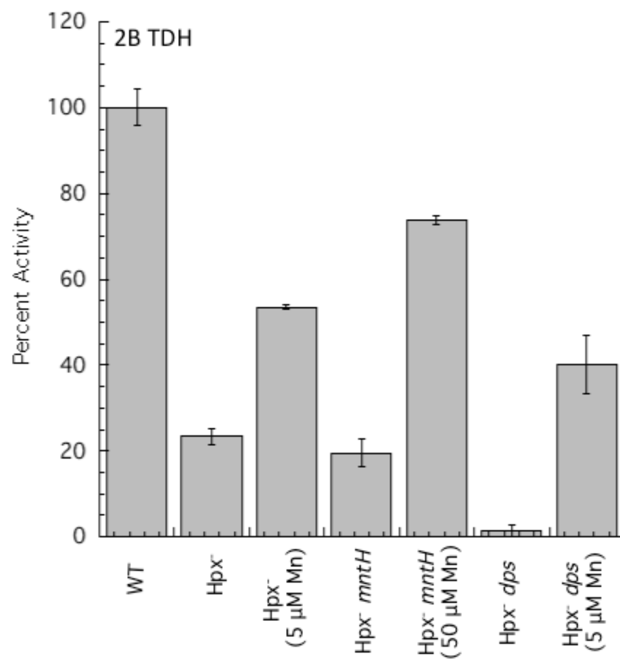


Figure 3.1. A. PDF, TDH and CDA are sensitive to H_2O_2 stress. Catalase deficient (*katE katG*) strains were grown aerobically in MinCAAG medium (Minimal A salts, glucose and Casamino Acids) and challenged with H_2O_2 *in vivo* (100 μM for 10 mins) or, *in vitro* (10 μM for 5 mins). B. Purified PDF was metallated with various transition metals (100 μM), and then challenged with H_2O_2 (10 μM for 5 mins). C. Purified TDH was metallated with various transition metals (100 μM), and then challenged with H_2O_2 (10 μM for 5 mins).

Figure 3.2





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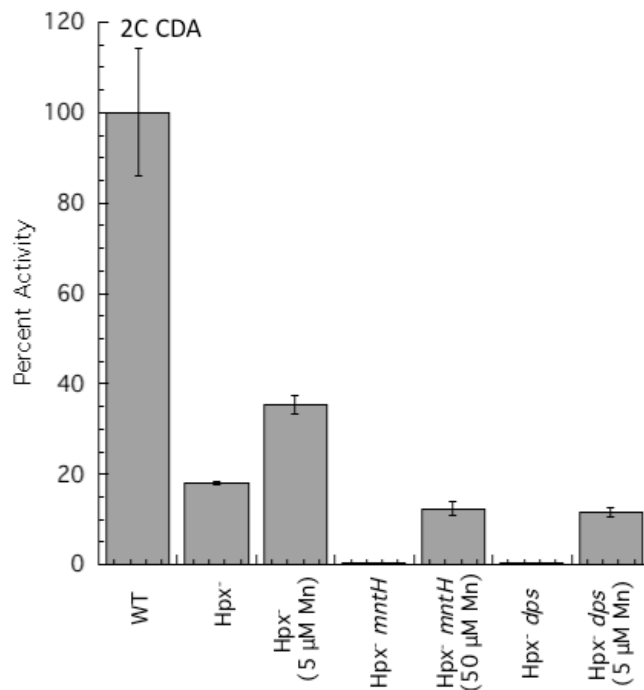


Figure 3.2. PDF, TDH and CDA are sensitive to H_2O_2 stress *in vivo*. Strains were grown aerobically in MinCAAG medium, with and without manganese supplementation. A. PDF activities, after *in vitro* metallation with nickel. B. TDH activities after *in vitro* metallation with iron. C. CDA activities after *in vitro* metallation with iron.

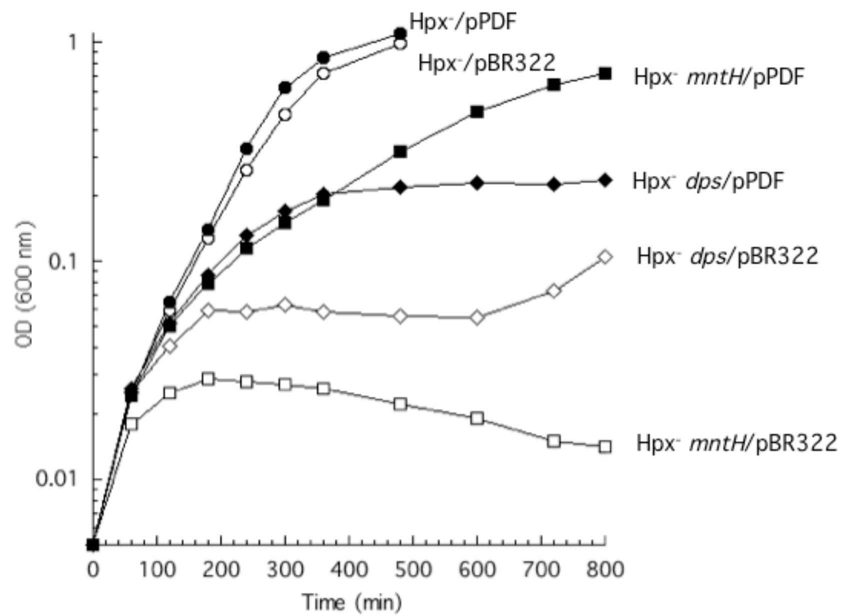


Figure 3.3. Overexpression of PDF relieves the Hpx- *mntH* growth defect. Cells were grown in MinCAAG medium in the presence of ampicillin, aerobically. Strains used were LC106/pBR322 (Hpx-, open circles), LC106/pPDF (Hpx-, closed circles), AA30/pBR322 (Hpx- *mntH*, open squares), AA30/pPDF (Hpx- *mntH*, closed squares), SP66/pBR322 (Hpx- *dps*, open diamonds) and SP66/pPDF (Hpx- *dps*, closed diamonds).

Figure 3.4

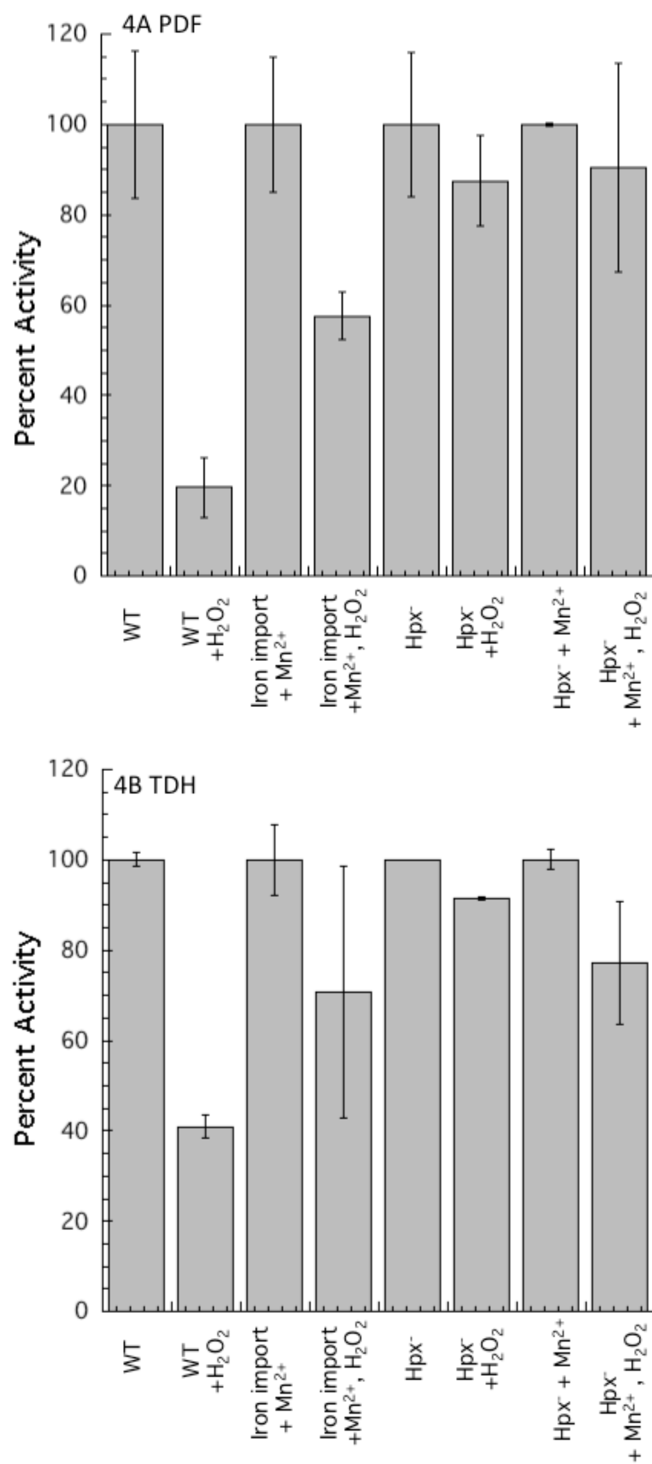


Figure 3.4 (Continued)

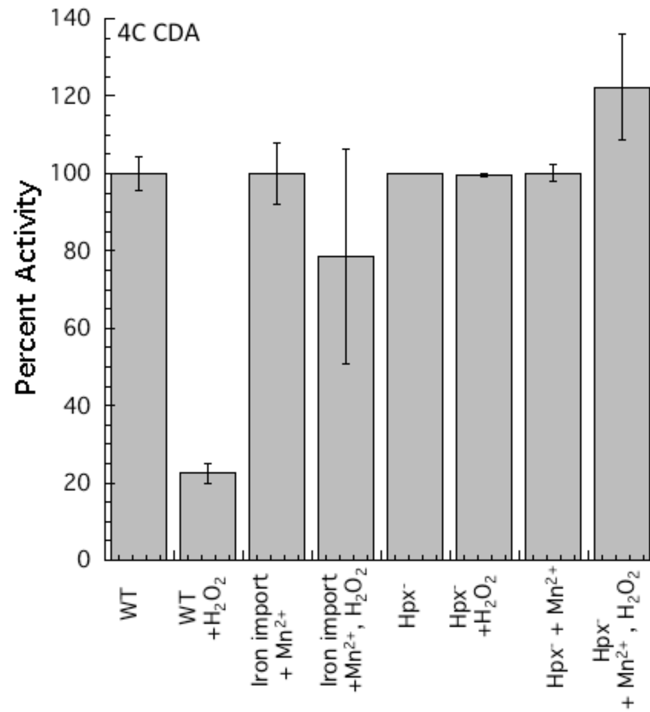
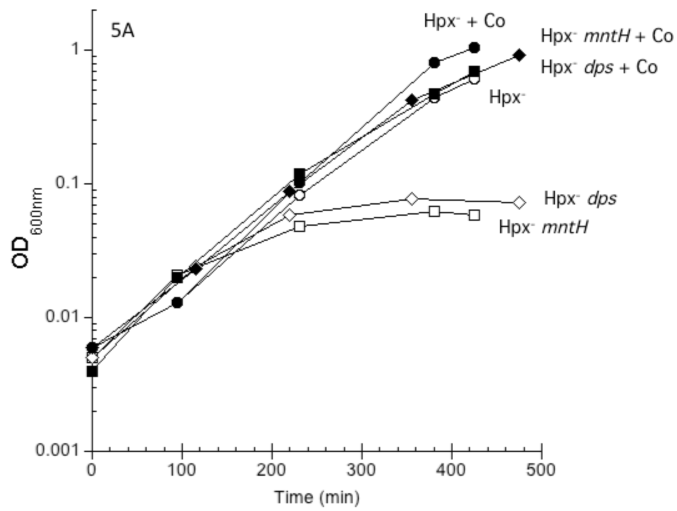


Figure 3.4. Manganese is able to prevent H_2O_2-mediated damage to PDF, TDH and CDA. Cells were grown in MinCAAG medium, aerobically. Cells were supplemented with 5μM $MnCl_2$ where indicated. In the presence of chloramphenicol, cells were exposed to 100 μM H_2O_2 for 10 mins. A. PDF activities, after *in vitro* metallation with nickel. B. TDH activities after *in vitro* metallation with iron. C. CDA activities after *in vitro* metallation with iron.



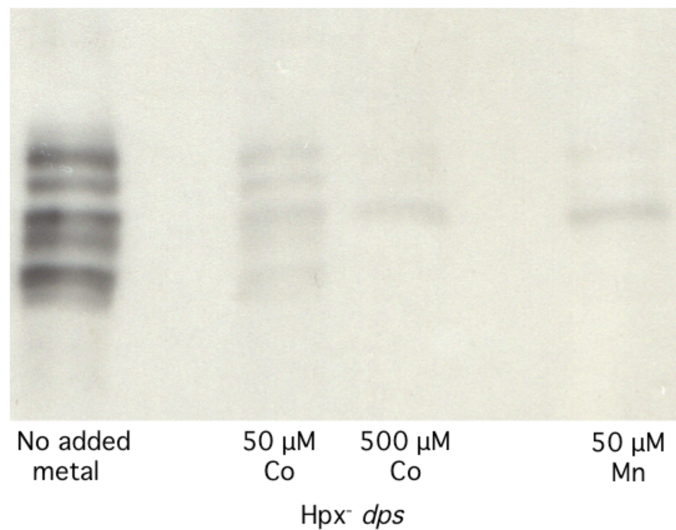
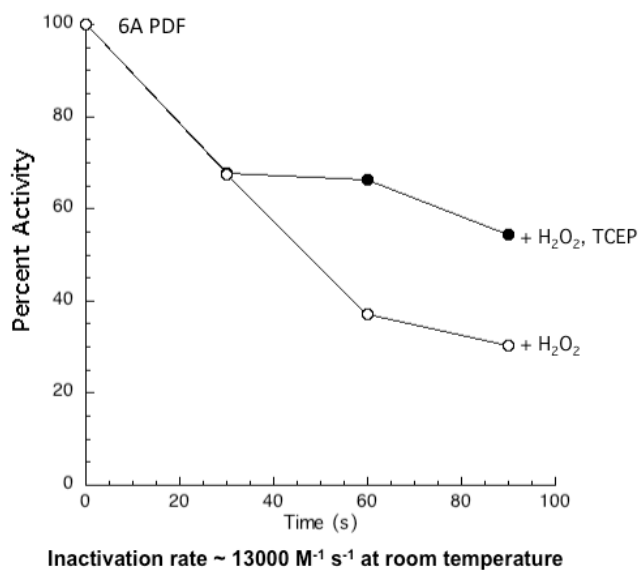


Figure 3.5. Cobalt supplementation also protects H_2O_2-stressed cells. A. Cobalt supplementation rescues both Hpx⁻ *mntH* and Hpx⁻ *dps* cells. Cells were grown in MinCAAG medium, aerobically. Strains used were LC106 (Hpx⁻, open circles), LC106 grown with 30 µM $CoCl_2$ (Hpx⁻, closed circles), AA30 (Hpx⁻ *mntH*, open squares), AA30 grown with 30 µM $CoCl_2$ (Hpx⁻ *mntH*, closed squares), SP66 (Hpx⁻ *dps*, open diamonds) and SP66 grown with 500 µM $CoCl_2$ (Hpx⁻ *dps*, closed diamonds). B. Hpx⁻ *dps* cells, grown with cobalt, have substantially lower levels of protein carbonylation.



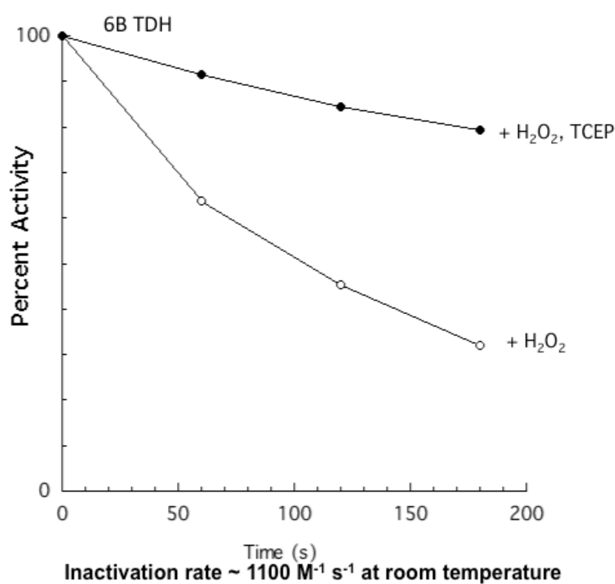
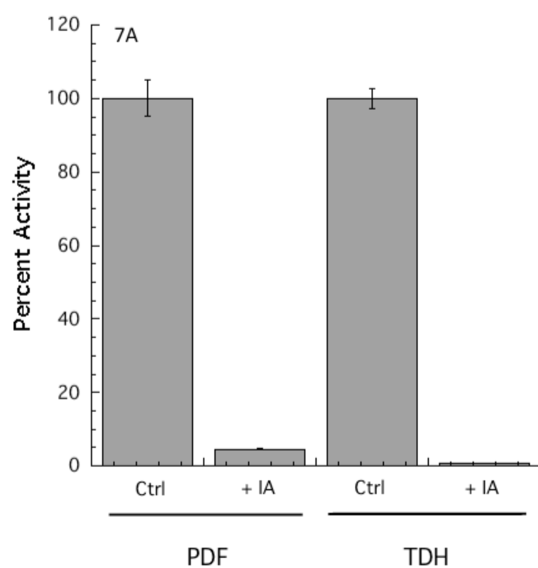


Figure 3.6. Apo-PDF and apo-TDH are sensitive to H_2O_2. Assays done at room temperature. A. Pure apo-PDF challenged with 1 μM H_2O_2, after which enzyme was repaired with 500μM TCEP, as indicated. Activity was measured in the presence of nickel. B. Pure apo-TDH challenged with 6 μM H_2O_2, after which enzyme was repaired with 500μM TCEP, as indicated. Activity was measured in the presence of zinc.



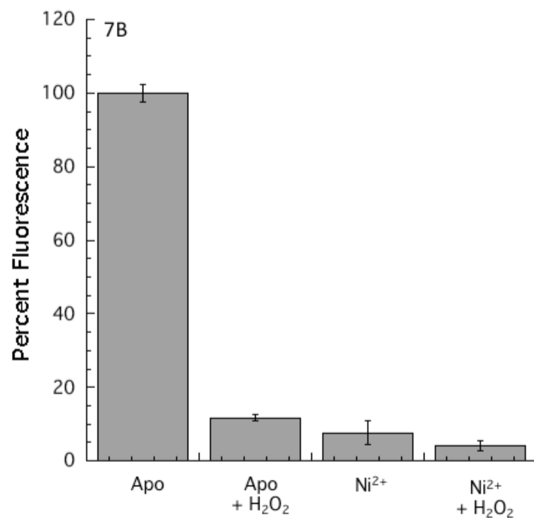


Figure 3.7. A. Iodoacetamide inactivates iron charged PDF and TDH. Pure PDF and TDH were metallated with ferrous iron, and then incubated with 25 mM iodoacetamide for 45 minutes at 37° C. The enzymes were then assayed. B. Pure Apo and nickel charged PDF were challenged with 20 μM H_2O_2 for 1 minute, after which the enzymes were incubated with 500 μM monobromobimane for 30 minutes. Fluorescence was then measured as described in *Experimental procedures*.

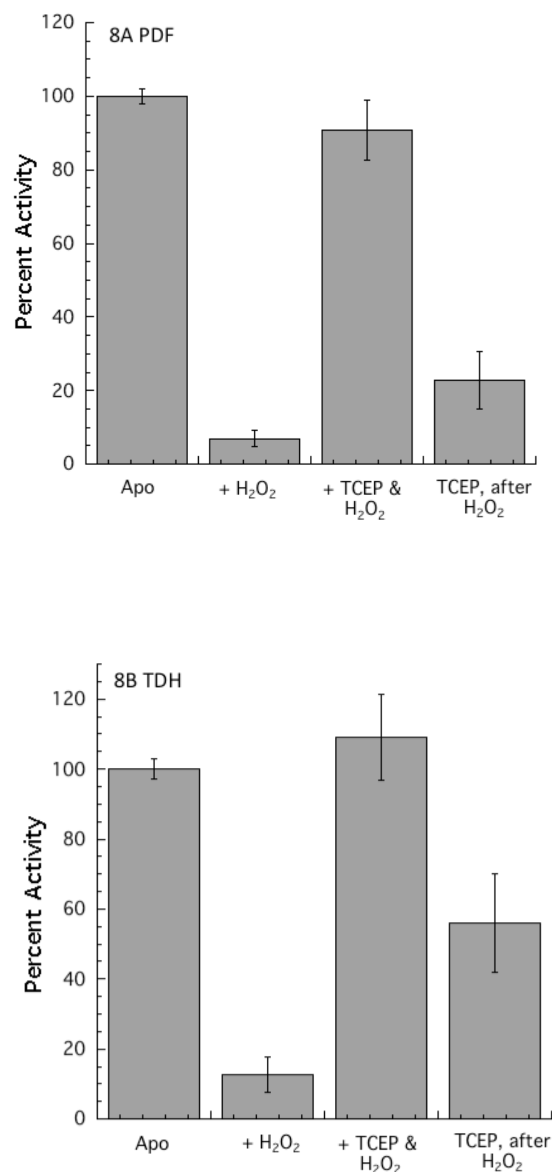
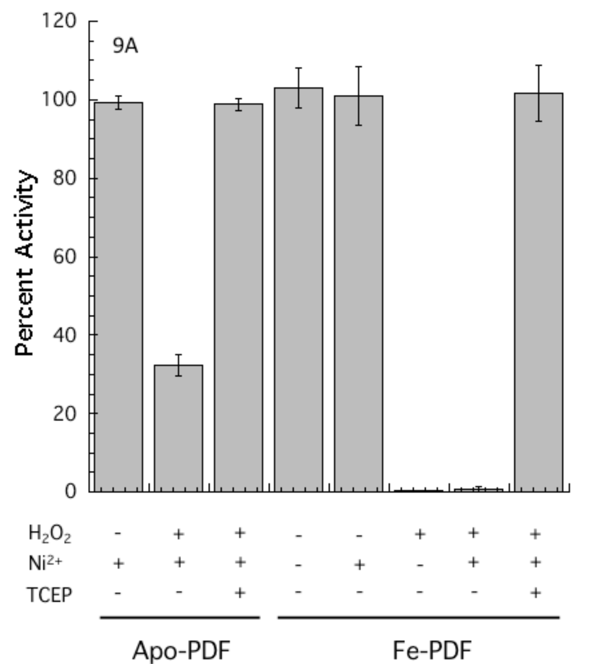


Figure 3.8. PDF and TDH can be overoxidized *in vitro*. A. Pure apo-PDF was exposed briefly to H_2O_2 (100 μM, 1 min), +/- 500 μM TCEP present during the exposure. Samples exposed to H_2O_2 without TCEP were later reduced with 500 μM TCEP as indicated. All samples were later assayed with nickel (500 μM). B. Pure apo-TDH was exposed briefly to H_2O_2 (100 μM, 1 min), +/- 500 μM TCEP present during the exposure. Samples exposed to H_2O_2 without TCEP were later reduced with 500 μM TCEP as indicated. All samples were later assayed with zinc (500 μM).



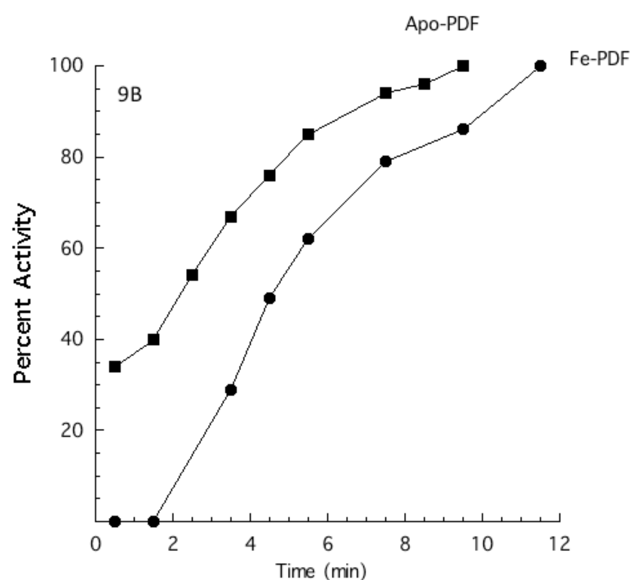


Figure 3.9. Cysteinyl residue in PDF active site is preferentially oxidized. A. H_2O_2 inactivation of pure iron-metallated PDF does not result in an apo enzyme. Catalase, nickel (500 μM) and TCEP (500 μM) were added after H_2O_2 exposure (50 μM, 5 seconds). B. Ferric iron leaves the active site within minutes. Pure iron-charged PDF was exposed to H_2O_2 (25 μM) in the presence of nickel (500 μM) and TCEP (500 μM), while nickel (500 μM) was added to apo-PDF after a 20 second H_2O_2 exposure (25 μM) with TCEP (500 μM) present from before.

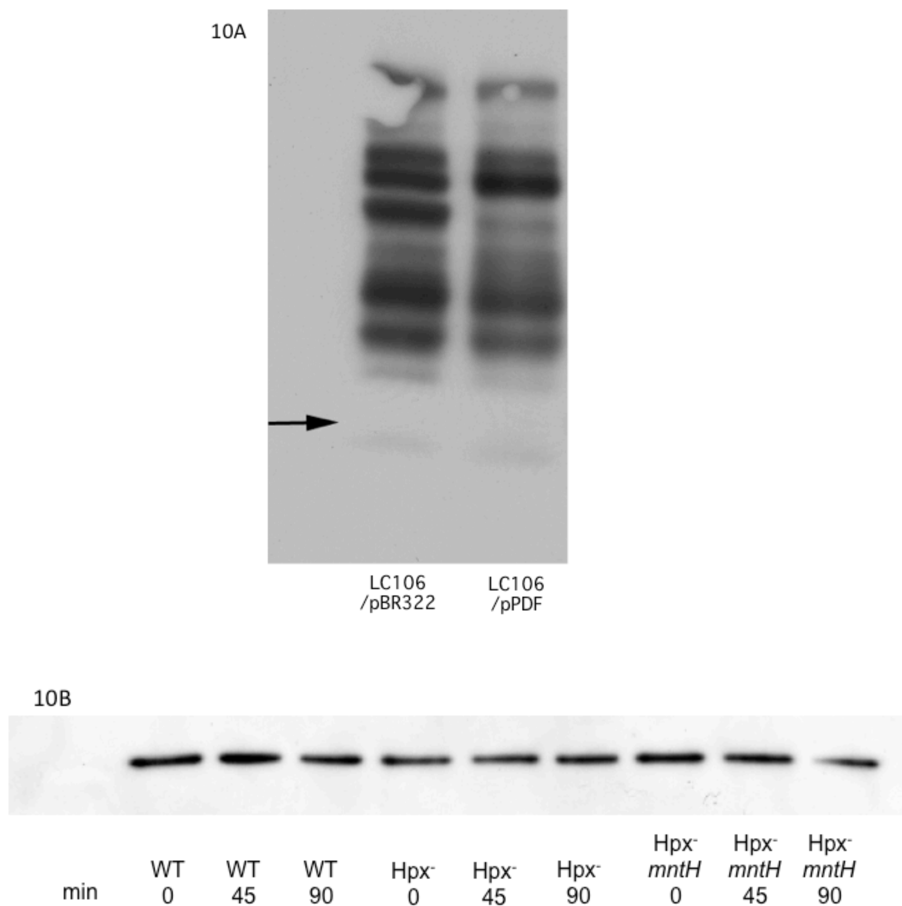


Figure 3.10. A. Peptide deformylase is not carbonylated *in vivo*. Hpx^- cells +/- PDF overexpression plasmid were grown in aerobic defined medium (glucose/amino acids), and proteins were harvested at an OD_{600} of ~ 0.2. Proteins were derivatized and Western blotted against DNPH as described in *Experimental procedures*. Strains used were LC106/pBR322 and LC106/pPDF. B. PDF polypeptide is not degraded *in vivo*. Cells expressing PDF-FLAG on a plasmid were grown anaerobically in defined medium (lactose/amino acids). After cells had reached an OD_{600} of ~ 0.2, chloramphenicol was added and cells were moved to air ($+O_2$). Presence of protein was tracked by Western blotting against FLAG tag. Strains used were MG1655/pPDF-FLAG, LC106/pPDF-FLAG (Hpx^-) and AA30/pPDF-FLAG (Hpx^- *mntH*).

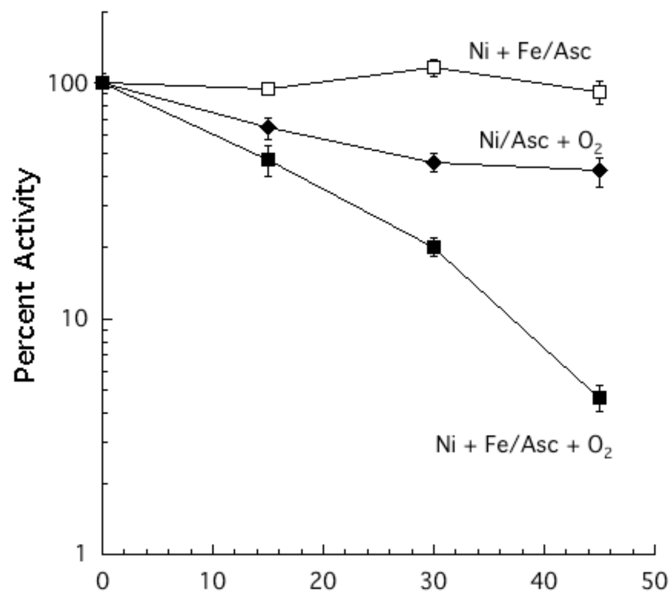


Figure 3.11. PDF can be irreversibly damaged by repetitive Fenton chemistry *in vitro*. Pure nickel or iron charged PDF were incubated in the presence of ascorbate aerobically and anaerobically. At defined time points, DTPA and catalase were added to terminate damage by ascorbate and iron aerobically, and PDF assayed in the presence of excess nickel.

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CHAPTER 4: CONCLUSIONS

4.1 SUMMARY OF CURRENT WORK

4.1.1 The role of manganese in protecting cells against reactive oxygen species

During routine growth *Escherichia coli* prefers to use iron as its primary metal cofactor. This is evident in that iron-containing superoxide dismutase (SOD) is the predominant SOD during routine growth, as opposed to manganese-containing SOD (MnSOD). When these cells are provided with sufficient manganese, the activity of MnSOD increases 20-fold. We have found that this low MnSOD activity is not due to repressed transcription, but rather due to incorrect metallation of MnSOD. This increased activity implies that, under normal growth conditions, *E. coli* does not import enough manganese even to metallate enzymes. This result suggests that perhaps cells do not depend upon manganese for normal metabolic function. We tested this hypothesis by deleting *mntH*, the only dedicated manganese transporter in *E. coli*. Mutants that lack MntH, the importer, grow at wild-type rates, indicating that manganese plays no critical role. However, MntH supports the growth of iron-deficient cells [10], suggesting that manganese can substitute for iron in activating at least some metalloenzymes. MntH is also strongly induced when cells are stressed by hydrogen peroxide. This adaptation is essential, as *E. coli* cannot tolerate peroxide stress if *mntH* is deleted. Other workers have observed that manganese improves the ability of a variety of microbes to tolerate oxidative stress, and the prevailing hypothesis is that manganese does so by chemically scavenging hydrogen peroxide and/or superoxide [1, 6, 7, 11, 13, 17]. We found that manganese does not protect peroxide-stressed cells by scavenging peroxide. Instead, the beneficial effects of manganese correlate with its ability to metallate mononuclear enzymes. Because iron-loaded enzymes are vulnerable to the Fenton reaction, the substitution of manganese may prevent protein damage. Accordingly, during H_2O_2 stress, mutants that cannot import manganese and/or are unable to sequester iron suffer high rates of protein oxidation.

4.1.2 Mechanism of manganese protection against ROS

In this study we demonstrated that three functionally distinct mononuclear enzymes, peptide deformylase (PDF), threonine dehydrogenase (TDH) and cytosine deaminase (CDA), use iron as their cofactor, and that manganese functionally replaces iron and therefore protects these enzymes when cells are stressed with H_2O_2. We used a literature approach to identify these enzymes, by testing enzymes that were known to use multiple transition metals, even if these enzymes were not known to use iron as a cofactor. We believe iron is frequently overlooked due to rapid oxidation of ferrous iron to the insoluble ferric form in aerobic buffers. There are over two hundred mononuclear enzymes in *E. coli*, quite a few of which could be using iron in non-stressed conditions and thus during H_2O_2-stressed conditions could be using manganese. This implies that damage to iron-loaded enzymes is a global problem for cells. As we have demonstrated before, manganese import is critical for cell survival under H_2O_2 stress. In fact, the key metabolic failure of Hpx^- *mntH* cells is due to lack of PDF activity: overexpressing PDF allows these cells to grow. We also demonstrated that these enzymes use manganese as their cofactor in iron-starved but H_2O_2-scavenging cells, which precludes the role of manganese as a scavenger. This conclusion is also supported by the ability of cobalt to relieve the cellular dependence on manganese during H_2O_2 stress.

4.1.3 Enzymes with metal-coordinating cysteinyl residues are prone to rapid oxidation

Both PDF and TDH have a metal-coordinating cysteine residue. We have demonstrated that in the apo-form both of these enzymes are sensitive to H_2O_2. While H_2O_2 is known to oxidize free cysteine, it does so at a maximal rate of $2\ M^{-1}\ s^{-1}$ at neutral pH [23], which is not fast enough to be physiologically relevant. In contrast, we have demonstrated that PDF and TDH are oxidized at a physiologically relevant rate of $13000\ M^{-1}\ s^{-1}$ and $1100\ M^{-1}\ s^{-1}$ respectively. These two enzymes are the first examples of H_2O_2 reacting with cysteine residues at such a fast rate. We

believe that cells use a very H_2O_2-senstive cysteine residue in these enzymes so that H_2O_2 preferentially oxidizes cysteine and therefore spares other protein residues from irreversible covalent damage. This oxidized cysteine can then be later repaired by a reductant. Consistent with this idea, we have shown that oxidized inactivated proteins can be easily restored to full activity by a reductant *in vitro*. We have also only been able to retrieve proteins with cysteinyl residues in the over-oxidized state, which implies that the cell repairs oxidized proteins quite rapidly *in vivo*.

4.2 POSSIBLE FUTURE WORK

4.2.1 Which enzyme is responsible for the aromatic amino acid auxotrophy?

Cells stressed with O_2^- or H_2O_2 (Jason Sobota, unpublished work) have been shown to be aromatic amino acid auxotrophs. Superoxide is thought to cause the aromatic amino auxotrophy by oxidizing the intermediate of the transketolase reaction [5, 21]. Manganese has been shown to relieve the aromatic amino acid auxotrophy, and workers have suggested that manganese does this by scavenging O_2^- [1]. In this study, we demonstrated that manganese supplementation also relieves the aromatic amino acid auxotrophy caused by H_2O_2 (Figure 4.1). However, we were able to show that mechanism of protection by manganese is not via scavenging H_2O_2 (Figure 4.2). Since manganese supplementation relieves the aromatic amino acid auxotrophy in both O_2^- and H_2O_2 stressed cells, it is likely that both O_2^- and H_2O_2 cause the auxotrophy in a similar manner. In this work, we have also shown that manganese does not act as a ROS scavenger *in vivo*. Therefore, it is likely that manganese relieves the auxotrophy by protecting mononuclear enzyme in the aromatic amino acid pathway in a manner similar to what we have seen with ribulose phosphate epimerase (RPE) [18], peptide deformylase (PDF), threonine dehydrogenase (TDH) and cytosine deaminase (CDA). Since transketolase, also a mononuclear enzyme, has recently been demonstrated to be resistant to H_2O_2 stress [18], it is unlikely that O_2^- damages this enzyme. Recent work by Jason Sobota has shown that activity of 2-dehydro-3-deoxyphosphoheptonate aldolase (AroF, AroG &

AroH), which is known to use iron as a cofactor [19], has significantly low activity in H_2O_2-stressed cells. Therefore, this enzyme might also by susceptible to damage by O_2^-.

As shown in this work, both peptide deformylase and threonine dehydrogenase use a cysteinyl residue to coordinate iron. Recent work by Mianzhi Gu has shown that both peptide deformylase and threonine dehydrogenase are sensitive to O_2^- stress *in vivo* and *in vitro*. While superoxide cannot oxidize cysteine directly, it could potentially do so indirectly by oxidizing iron and thus could damage these enzymes. Interestingly, 2-dehydro-3-deoxyphosphoheptonate aldolase also uses at least a cysteinyl residue to coordinate metal in the active site [20]. Therefore damage to the cysteine residue(s) on this enzyme could explain the aromatic amino acid auxotrophy caused by both O_2^- and H_2O_2.

The enzyme activity of the aldolase could be tested during O_2^- and H_2O_2 stress to see if the enzyme is susceptible to damage. If the aldolase is vulnerable, overexpression of the enzyme in O_2^- and H_2O_2 stressed cells might be able to relieve the auxotrophy. It would be worthwhile to measure the rate of apo-enzyme inactivation to check if the cysteinyl residue is indeed sensitive to ROS, and if so does the rate of inactivation compare to that of apo-PDF and apo-TDH. Other mononuclear enzymes could be tested not only for their susceptibility to ROS but also if cysteine is a residue of choice in the active site of these enzymes. Perhaps analysis of other such enzymes might elucidate a pattern that could help explain why some mononuclear enzymes use a ROS sensitive residue in their active sites.

4.2.2 How does manganese relieve the leucine auxotrophy?

Low micromolar levels of H_2O_2 have been shown to block the leucine amino acid pathway by damaging isopropylmalate isomerase (IPMI), a solvent-exposed iron-sulfur-cluster-containing enzyme [12]. We have shown that manganese supplementation can relieve the leucine amino acid auxotrophy (Figure 4.3). Isopropylmalate isomerase activity is also almost fully active in these manganese-

supplemented H_2O_2-stressed cells (Figure 4.4). It is improbable that manganese replaces the iron in iron-sulfur clusters: no such *in vivo* examples are known to exist and the clusters are designed specifically to work with iron. However, it might be possible considering similar mangano-clusters have been shown to be stable *in vitro* [4, 15, 24]. It is not clear if such structures would be functional *in vivo*, nor would they necessarily need to be: by occupying the solvent exposed iron site, manganese could prevent the cluster from disintegrating or from further damage, and thus provide cellular repair processes enough time to recover the enzyme fully. So far, we have not seen a protective effect by manganese on iron-sulfur clusters *in vitro*: we have checked to see if manganese can replace iron in solvent exposed iron-sulfur clusters and if the presence of manganese protects such enzymes against H_2O_2 damage.

It is more likely that protective effect of manganese on iron-sulfur clusters is indirect: manganese supplementation spares iron for other processes, which would allow the cell to more quickly repair its damaged iron-sulfur cluster enzymes.

It might also be possible that damage to an iron-utilizing mononuclear enzyme is responsible for the leucine auxotrophy, as opposed to damage to IPMI. However, the leucine biosynthesis pathway has no such known obvious candidates.

4.2.3 Do iron-independent organisms metallate mononuclear enzymes with manganese?

Organisms such as *Lactococcus plantarum* [2, 3] and *Borrelia burgdorferi* [8, 16] have iron-independent metabolisms and import little to no iron. These organisms also import high concentrations of manganese, up to a millimolar in *Lactococcus plantarum*. It is likely that manganese predominantly metallates mononuclear enzymes in these organisms, which might be one of the main reasons that these organisms are resistant to ROS stress.

It is generally thought that perhaps these organisms evolved from iron dependency because of the scarcity of iron in their environments. For instance, *Borrelia burgdorferi* is a pathogen that is dependent on its host, such as humans, for survival. Our immune systems, however, produce iron chelators as a possible mechanism to suppress pathogen growth. Therefore, iron independence would help *Borrelia burgdorferi* to circumvent our immune system. Another reason why these organisms evolved iron-independent metabolisms might be because iron-dependent organisms are vulnerable to ROS stress. In the case of *Lactococcus plantarum*, the organism takes advantage of this vulnerability by releasing H_2O_2 in its environment to toxify its competitors and thereby monopolizes other nutrients in its environment.

So why have other organisms also not moved away from iron dependency? While manganese can replace iron in mononuclear enzymes, it cannot do so for iron-dependent redox enzymes and iron-sulfur clusters. Even though mangano-dependent redox isozymes exist, manganese is not known to replace the role of iron in iron-sulfur clusters. Prototrophic organisms depend on iron-sulfur cluster-utilizing enzymes for biosynthesis, which could explain why organisms continue to tolerate the ROS-dependent vulnerability associated with iron usage.

It would be interesting to check if organisms such as *Lactococcus plantarum* and *Borrelia burgdorferi* do indeed use manganese to metallate their mononuclear enzymes. The sensitivity of these enzymes towards ROS could be checked in crude extracts. If so, is it because the enzymes non-specifically bind to the most abundant metal or do they have a high affinity for manganese? Are other proteins involved that help maintain manganese in these enzymes? Mononuclear enzymes from these organisms could be expressed in *E. coli* to help explain how these enzymes are metallated in their respective organisms. If these enzymes are functional in *E. coli* and use iron, then these enzymes are metallated by the most predominant metal in the cytoplasm. However, if these enzymes are continued to be metallated by manganese, then studying their affinity could help explain metal specificity for mononuclear enzymes in general.

4.2.4 Do eukaryotes continue to metallate mononuclear enzymes with iron?

If eukaryotes use iron to metallate mononuclear enzymes, it would be interesting to see if ROS related damage to these enzymes could help explain any known human diseases. Do eukaryotic organisms also depend on manganese to functionally replace iron in these enzymes, when stressed with ROS? This might be possible, considering manganese supplementation has been shown to have a protective effect in ROS-stressed yeast [17] and in *Caenorhabditis elegans* [14]. Threonine dehydrogenase, cytosine deaminase and ribulose phosphate epimerase activities could be assayed in ROS-stressed and non-stressed yeast to check if these enzymes are vulnerable to damage. Also, yeast homologs of these enzymes could be expressed in *E. coli* to check if iron or manganese metallates these enzymes. The answer either way would be interesting: if the enzymes are metallated with iron then mononuclear enzymes of eukaryotes are susceptible to damage. However, if these enzymes are metallated with manganese then that would imply that eukaryotes have evolved to minimize damage to their mononuclear enzymes.

4.2.5 How are cysteinyl-residues re-reduced *in vivo*?

In this study we have shown that both PDF and TDH have H_2O_2-sensitive sulfhydryls in their active sites. These enzymes can be oxidized by H_2O_2 *in vitro* and repaired by a reductant, such as TCEP, unless the enzymes are over-oxidized. However, these enzymes are only found in the over-oxidized state in cells treated with H_2O_2 *in vivo*. This suggests that oxidized cysteinyl-residues in these enzymes are quickly reduced *in vivo*: over-oxidized sulfhydryls seem to be non-repairable. What is responsible for reducing these cysteinyl-residues inside the cells? Both glutaredoxin 1 and thioredoxin 2, which are induced by OxyR, seem to have no effect on these enzymes (Figure 4.5). So far workers have been unable to show why cells require the induction of glutaredoxin 1 and thioredoxin 2 in H_2O_2-stressed cells. Perhaps other reducing agents, such as glutaredoxin 2 and thioredoxin 1 can compensate for glutaredoxin 1, 3 and 4, and thioredoxin 2 and 3. Glutathione might

also be responsible, although preliminary results suggest otherwise (data not shown). To understand how PDF and TDH are reduced *in vivo*, most of these reducing proteins would need to be knocked out, if possible.

Since both apo-PDF and apo-TDH can be over-oxidized, a question remains as to if these over-oxidized enzymes can be repaired *in vivo*. Cells, in the presence of chloramphenicol, could be exposed to a brief but high dose of H_2O_2 (100 μM), and then PDF and TDH activities could be assayed over time. If these over-oxidized enzymes are indeed repairable *in vivo*, then PDF and TDH activities should increase over time. The rate of repair could be calculated to understand the importance of this type of repair. It would also be interesting to understand the process by which such repair happens.

4.2.6 Why do cells require the induction of GpmM during H_2O_2 stress?

E. coli has two phosphoglycerate mutase isozymes: cofactor-dependent and cofactor-independent. GpmA, the cofactor-dependent isozyme, is the primary mutase of the cell. While GpmA is not a mononuclear enzyme, it is curiously regulated by Fur. However GpmM, the cofactor-independent mutase, is a mononuclear enzyme and uses manganese for activity [9, 22]. We have shown that cells deleted for GpmM in H_2O_2-stressed cells undergo a severe growth lag (Figure 4.6). This suggests that GpmA function is hampered under these conditions, and perhaps GpmA is getting damaged. It is not clear how this might be happening. Also, why is GpmA regulated by Fur? Perhaps this enzyme does require a metal for activity? This could be easily tested by assaying the purified enzyme in the presence of various metals. This experiment should also be done in the presence or absence of concentrated crude extract: perhaps a mutase-activating enzyme requires metals for activity. Therefore, the presence of metal would not activate the mutase more efficiently. Cofactor dependent phosphoglycerate mutases require 2,3-bisphosphoglycerate for activity and it is not clear how this cofactor is made in *E. coli*.

4.2.7 How does manganese continue to be transported in to *mntH* cells?

We have observed that *mntH* mutants are still able to metallate a small percentage of manganese-dependent superoxide dismutase. Hpx^- *mntH* mutants can also be rescued by supplementing cells with exogenous manganese. Is there another manganese transporter in *E. coli*? The answer to this would be interesting considering *E. coli* lacks the other dedicated manganese transporter found in other organisms, *mntABC*. It is possible that manganese adventitiously enters the cell via other metal importers. However, manganese is still able to enter *mntH* cells even if cells are mutated for iron transporters.

4.3 FIGURES

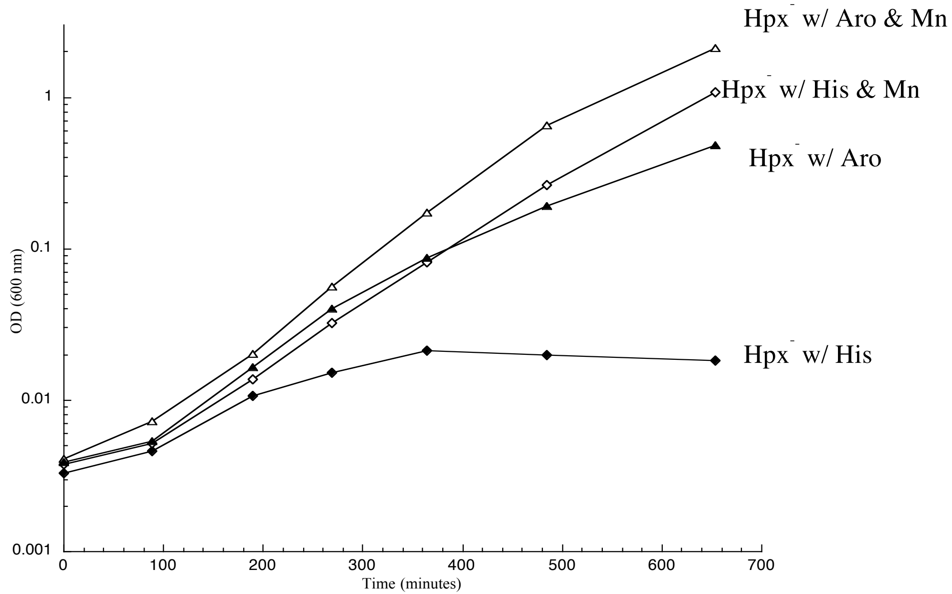

Figure 4.1. Manganese relieves the aromatic amino acid auxotrophy. Cultures were grown to log phase in anaerobic defined medium (minimal A salts, thiamine, glucose, and histidine). Cells were then diluted to 0.0025 OD_{600} in the same medium (with or without aromatic amino acid supplementation), manganese added to specific cultures as indicated (100 μM) and grown aerobically at 37°C.

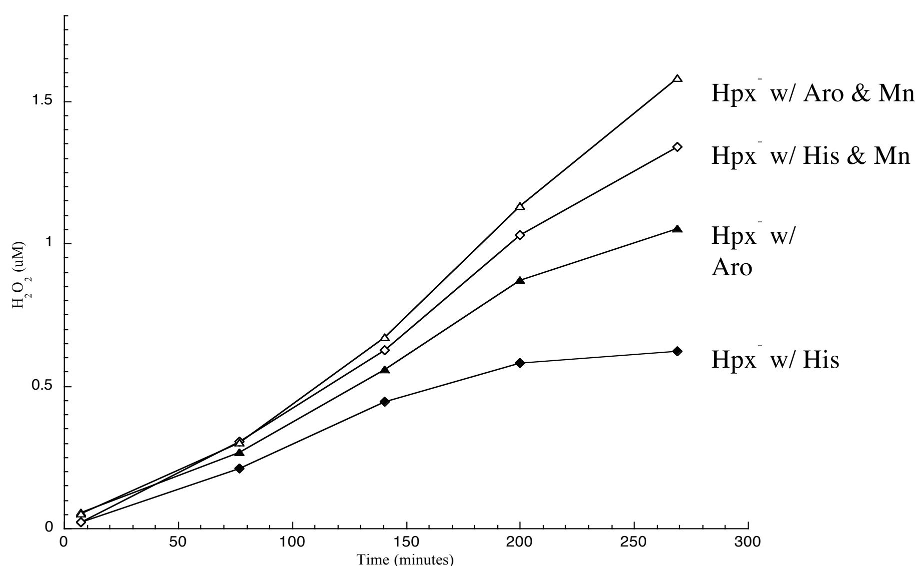

Figure 4.2. Manganese does not relieve aromatic amino acid auxotrophy by preventing H_2O_2 accumulation. Cultures were grown to log phase in anaerobic defined medium (minimal salts, thiamine, glucose and histidine). Cells were pelleted and resuspended in fresh defined medium, with or without aromatic amino acids (0.5 μM). Aerobic cultures were inoculated to 0.02 OD_{600} in fresh defined medium with manganese added as indicated (100 μM). Aliquots were periodically removed, centrifuged and hydrogen peroxide concentration was assayed in the supernatant. Cells were grown aerobically at 37°C.

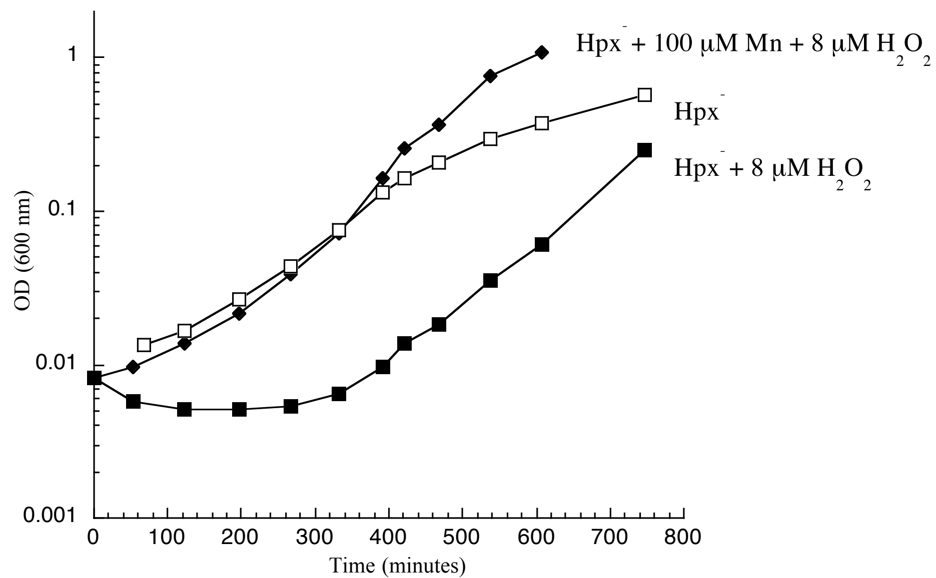

Figure 4.3. Manganese fed Hpx$^-$ cells do not exhibit leucine auxotrophy. Cultures were grown to log phase in anaerobic defined medium (minimal salts, thiamine, glucose, histidine and aromatic amino acids). At time zero cells were diluted to 0.008 OD_{600} into the same medium and 8 µM H_2O_2 was added, with and without manganese supplementation. These were then aerated at 37°C.

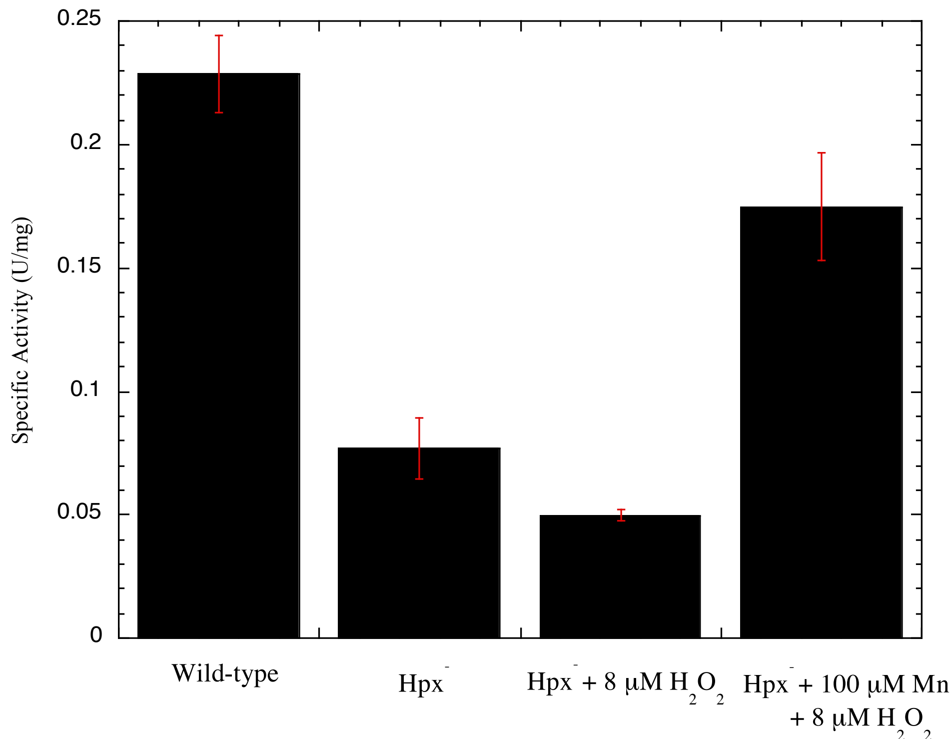

Figure 4.4. Manganese fed cells have high IPMI activity. Cultures were grown to 0.2 OD_{600} in anaerobic defined medium (minimal salts, thiamine, glucose, histidine and aromatic amino acids). These cultures were washed and resuspended in fresh defined medium. These new cultures were then moved to air and 8 μM H_2O_2 was added, with and without manganese supplementation. The cultures were harvested anaerobically after 40 minutes. IPMI activity was then measured. Error bars represent standard deviation of multiple measurements of the same sample.

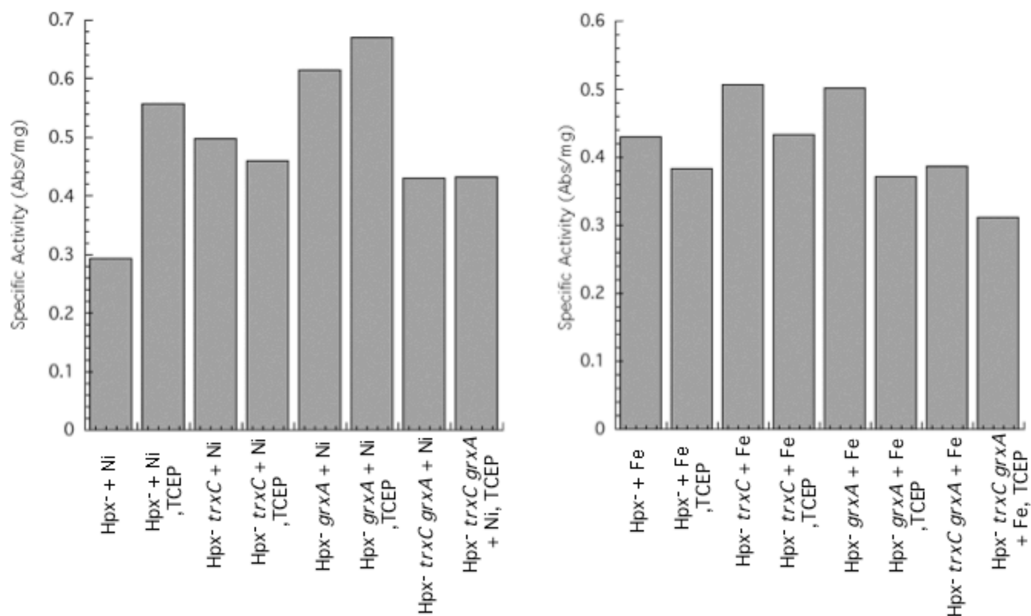

Figure 4.5. GrxA and TrxC are not involved in keeping the active site cysteine residue in PDF and TDH reduced in Hpx$^-$ cells. Cultures were grown aerobically in defined medium (glucose + Casamino Acids) to OD_{600} of ~0.2-0.3. These cells were then harvested and assayed for PDF and TDH activity. PDF was assayed in the presence of 500 μM nickel +/- 500 μM TCEP in crude extracts, while TDH was assayed in the presence of 500 μM ferrous iron +/- 500 μM TCEP in crude extracts.

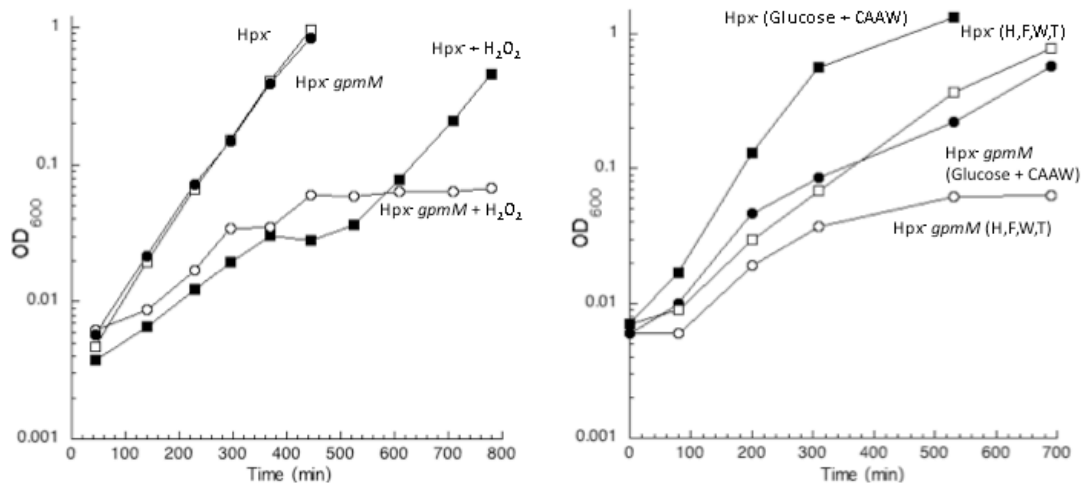

Figure 4.6. Manganese-dependent phosphoglycerate mutase is important during H_2O_2 stress. A. Cells were grown aerobically in defined medium (Glucose + Casamino Acids), +/- 20 μM H_2O_2. Hpx^- *gpmM* mutants, stressed with additional H_2O_2, go through a very long lag phase. B. Hpx^- *gpmM* are unable to grow in the presence of aromatic amino acids. Cells were grown aerobically in defined medium (Glucose) with either Casamino Acids or histidine and aromatic amino acids.

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Anjem A., Varghese S.M, Imlay J. (2009) *Manganese import is a key element of the OxyR response to hydrogen peroxide in Escherichia coli*. Mol Microbiol. 2009 May;72(4):844-58

- Subject of special commentary, McEwan AG. (2009) *New insights into the protective effect of manganese against oxidative stress.* Mol Microbiol. 2009 May;72(4):812-14
- Selected as article of special interest, Faculty of 1000 Biology: http://f1000biology.com/article/id/2555967/evaluation

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2009-2010	Member of Graduate and Professional Affairs Committee