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Superoxide poisons mononuclear iron enzymes by causing mismetallation

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Summary

Superoxide (O_2^-) is a primary agent of intracellular oxidative stress. Genetic studies in many organisms have confirmed that excess O_2^- disrupts metabolism, but to date only a small family of [4Fe-4S] dehydratases have been identified as direct targets. This investigation reveals that in *Escherichia coli* O_2^- also poisons a broader cohort of non-redox enzymes that employ ferrous iron atoms as catalytic cofactors. These enzymes were inactivated by O_2^- both in vitro and in vivo. Although the enzymes are known targets of hydrogen peroxide, the outcome with O_2^- differs substantially. When purified enzymes were damaged by O_2^- in vitro, activity could be completely restored by iron addition, indicating that the O_2^- treatment generated an apoprotein without damaging the protein polypeptide. Superoxide stress inside cells caused the progressive mismetallation of these enzymes with zinc, which confers little activity. When O_2^- stress was terminated, cells gradually restored activity by extracting zinc from the proteins. The overloading of cells with zinc caused mismetallation even without O_2^- stress. These results support a model in which O_2^- repeatedly excises iron from these enzymes, allowing zinc to compete with iron for remetallation of their apoprotein forms. This action substantially expands the physiological imprint of O_2^- stress.

Introduction

Superoxide dismutase (SOD) was discovered fortuitously as a trace contaminant that inhibited the reduction of cytochrome *c* by enzymatically generated superoxide (O_2^-) (McCord and Fridovich, 1969). The ubiquity of SOD among aerobic organisms suggested that O_2^- is a regular hazard of the aerobic lifestyle (McCord *et al.*, 1971). It was quickly demonstrated that O_2^- can be formed by the accidental autoxidation of redox enzymes (Massey *et al.*, 1969); however, workers struggled to identify biomolecules that O_2^- might damage (Bielski and Richter, 1977; Fee, 1982; Fitzsimons, 1979; Sawyer and Valentine, 1981). It does not react at significant rates with amino acids, nucleic acids, lipids, or carbohydrates. The ability of O_2^- to reduce ferric iron led to the proposal that it might drive Fenton reactions, which generate the hydroxyl radicals that oxidize proteins and DNA (McCord and Day, 1978). However, while this chemistry succeeded in vitro, the concentration of O_2^- in vivo is far too low to damage cells in this way (Keyer and Imlay, 1996).

This dilemma was addressed by the creation of *E. coli* mutants that lack its cytoplasmic iron- and manganese-cofactored SODs. These strains grow at normal rates under anaerobic conditions, but in aerobic medium they are unable to synthesize some amino acids, cannot catabolize TCA-cycle substrates, and exhibit high rates of spontaneous mutagenesis (Carlioz and Touati, 1986; Farr *et al.*, 1986). In 1987 Kuo and Fridovich announced that excess O_2^- blocks the synthesis of branched-chain amino acids by inactivating dihydroxyacid

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dehydratase, an enzyme in the common pathway for Leu, Ile, and Val biosynthesis (Kuo *et al.*, 1987). This enzyme employs a solvent-exposed $[4\text{Fe-4S}]^{2+}$ cluster that directly binds and activates substrate. Aconitase and fumarases A and B of the TCA cycle use similar catalytic mechanisms, and their inactivation underlies the inability of SOD mutants to grow on TCA-cycle substrates (Gardner and Fridovich, 1991; Liochev and Fridovich, 1993). In each case, O_2^- rapidly oxidizes the cluster to an unstable $[4\text{Fe-4S}]^{3+}$ valence; it then spontaneously releases Fe^{2+} , collapsing to an inactive $[3\text{Fe-4S}]^+$ cluster (Flint *et al.*, 1993). The same enzyme family is found throughout the biota, including in human cells, and its damage is now widely recognized as contributing to the pathology of oxidative stress.

However, other consequences of O_2^- stress are not fully understood. *E. coli* and yeast SOD⁻ mutants contain high levels of intracellular free iron and thereby suffer marked Fenton-dependent DNA damage, as evidenced by their high rates of mutagenesis (Farr *et al.*; 1986, Keyer and Imlay, 1996; Srinivasan *et al.*, 2000). The excess free iron may derive in part from iron leakage from the degraded dehydratase clusters. However, the iron levels remain high even when *E. coli* SOD⁻ mutants are cultured in media supplemented with glucose and amino acids, in which these dehydratases are largely repressed. Further, under these conditions growth is not robust (Imlay and Fridovich, 1992). These observations suggest the existence of other mechanisms by which O_2^- can compromise metabolism and disrupt iron homeostasis. In this study we pursued the basis of these effects. We report that O_2^- disables non-redox mononuclear iron proteins by triggering their mismetallation. These proteins have wide-ranging roles in metabolism, and so the impact of this damage may be substantial.

Results

Endogenous superoxide disrupts Fur metallation

SOD⁻ mutants ($\Delta\text{sodA } \Delta\text{sodB}$) of *Escherichia coli* lack both cytosolic superoxide dismutases (SODs) and therefore accumulate toxic amounts of intracellular superoxide (O_2^-). We observed that in aerobic glucose/amino acids medium, iron-import genes such as *fhuA* and *iucC* were repressed in the wild type strain but fully expressed in the SOD⁻ strain (Fig. 1A). These genes are normally repressed under iron-sufficient conditions because the Fur repressor, in a complex with ferrous iron, binds and occludes their promoter region. When iron is scarce, apo-Fur protein dissociates from binding sites and iron-import genes are expressed (Lee *et al.*, 2007). The expression of these genes in SOD⁻ mutants indicated that Fur protein was demetallated. One explanation might be that there was not enough unincorporated iron in the SOD⁻ strain to metallate Fur. However, whole-cell EPR data showed that the SOD⁻ strain actually had a higher intracellular free iron level than did the wild type strain (Fig. 1B), confirming prior reports (Keyer and Imlay, 1996).

An alternative possibility was that O_2^- disrupts Fur metallation. Although O_2^- is widely recognized to be a reductant of Fe(III), it can also oxidize Fe(II) in some circumstances, such as in the catalytic cycle of iron-containing superoxide dismutase (Miller, 2012). Since Fur acts as a repressor only when it is bound by Fe(II), it seemed plausible that O_2^- oxidizes Fe(II) to its ferric form, which then dissociates from the metal-binding site of Fur and leaves it as an inactive apo-protein.

Technical hurdles make it challenging to fully test this model with Fur protein itself. However, these results raised the larger possibility that O_2^- might disrupt other ferrous-iron dependent proteins, including mononuclear iron enzymes. This type of enzyme employs a single Fe(II) atom as the catalytic cofactor. This Fe(II) is exposed to solvent within the active site. It does not change its oxidation state during catalysis, and its overall role is to help with substrate binding and to provide a local positive charge to stabilize the anionic reaction intermediate. Several such enzymes can be inactivated by hydrogen peroxide

(H₂O₂) through a metal-centered Fenton reaction (Sobota and Imlay, 2011, Anjem and Imlay, 2012). We decided to examine whether O₂⁻ inactivates these enzymes as well.

Mononuclear iron enzymes are damaged in the SOD-deficient strains

We selected threonine dehydrogenase (Tdh), ribulose-5-phosphate 3-epimerase (Rpe), and peptide deformylase (Pdf) for our study. These three enzymes catalyze different categories of non-redox chemical reactions, but they each employ a bound catalytic iron atom that coordinates substrate. All three activities were substantially lower in the SOD⁻ strain than in the wild-type strain (Fig. 2A). Further, flux through the Rpe-dependent pentose-phosphate pathway was progressively impeded, as shown by the poor growth of an SOD⁻ *edd*⁻ mutant when gluconate was supplied as the carbon source (Fig. 2B). This phenotype is consonant with damage to Rpe.

Superoxide rapidly inactivates mononuclear iron enzymes in vitro

To determine whether O₂⁻ can directly inactivate these mononuclear iron enzymes, the enzymes were purified. Native metals dissociate during this process, and so the purified proteins were stripped of adventitious metals by treatment with chelators and then loaded with ferrous iron. The enzymes were then exposed to O₂⁻ that was generated enzymatically by xanthine oxidase. Catalase was included in the reaction mix to prevent the accumulation of H₂O₂. All three iron enzymes rapidly lost activity (Fig. 3). The addition of SOD completely protected Tdh and Rpe, which confirmed that O₂⁻ directly inactivated these two enzymes.

Addition of Fe²⁺ fully restored activity to O₂⁻-damaged Tdh and Rpe, indicating that O₂⁻ treatment generated an apo-protein without any detectable damage to the polypeptide (Fig. 4). This result contrasts with the outcome when such enzymes are damaged by H₂O₂. The H₂O₂ reaction generates a ferryl radical. In Rpe this radical dissociates into a hydroxyl radical that damages the local polypeptide, while in Tdh it directly oxidizes the metal-coordinating cysteine ligand (Anjem and Imlay, 2012). In the latter case reduction of the resultant sulfenic acid is necessary for re-metallation to occur. In contrast, oxidation of the iron atom by O₂⁻ evidently does not result in cysteine oxidation.

SOD only partially protected Pdf from the treatment. We found that molecular oxygen itself also gradually inactivated Pdf (Fig. 3C); the rates at which Pdf lost activity correlated with the amounts of O₂ present in the assay (Fig. S2). To avoid this complication, our subsequent analyses focused on Tdh and Rpe.

The rate constants for enzyme inactivation were determined. Because O₂⁻ is highly unstable and its steady-state concentration cannot easily be measured, a competition assay system was used (see Experimental procedures). The rate constants were determined to be $1.6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Tdh) and $0.8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Rpe), which are comparable to those with which O₂⁻ reacts with [4Fe-4S] cluster dehydratases ($2\text{--}6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$). The steady-state concentration of O₂⁻ in wild type cells has been estimated to be ~0.2 nM (Imlay and Fridovich, 1991b), implying a half-time for the oxidation of those mononuclear iron enzymes by O₂⁻ of ~30 min.

SOD⁻ mutants exhibit high mutation rates due to their substantially elevated levels of intracellular iron (Farr *et al.*, 1986; Keyer and Imlay, 1996; Liochev and Fridovich, 1994). In part the extra iron may result from the damage to [4Fe-4S] dehydratases; however, the iron level remains high even in glucose/amino acids medium, a condition under which the dehydratases are metabolically unnecessary and at low titers (Fig. 1B). The effect is not fully due to Fur demetallation either, since the SOD⁻ *fur* strain had even higher iron level than the *fur* mutant alone. Therefore, it seemed plausible that the leaching of iron from

mononuclear iron enzymes during O_2^- stress might also contribute to the pool of free iron. Indeed, the level of free iron in the SOD^- strain was further elevated when Rpe was overproduced. Thus these enzymes likely contribute to the high level of unincorporated iron in O_2^- -stressed cells and, accordingly, to their vulnerability to Fenton chemistry.

In vivo, superoxide damages mononuclear iron enzymes by enabling mismetallation

To track the inactivation of Tdh in vivo, wild-type and SOD^- strains were grown anaerobically, protein synthesis was stopped by the removal of essential amino acids, and cultures were then aerated. At intervals, aliquots were returned to the anaerobic chamber, and lysates were quickly prepared and assayed. Over two hours Tdh progressively lost ~80% of activity; longer incubation did not decrease activity any further (Fig. 5A). The inactivation process appeared to be surprisingly slow *in vivo*, given the fact that Tdh lost > 90% activity within 3 min upon O_2^- -treatment *in vitro* (Fig. 3A). However, this slowness matched the gradual onset of growth problems (Fig. 2B).

The loss of Tdh activity could be explained in any of three, non-exclusive ways: 1) the enzyme might be converted to the un-metallated apo-protein form; 2) the protein polypeptide might be degraded; 3) or the enzyme might become mischarged with metals other than iron. We tested these three possibilities in turn.

The addition of metals to the extracts did not result in an increase in Tdh activity (Fig. 5B), indicating that they did not contain significant apo-Tdh. However, full activity was recovered when the extracts were incubated with EDTA prior to the iron addition (Fig. 5C). The latter result established that Tdh protein polypeptide was not degraded in the SOD^- strain, and it implied that the Tdh active sites were occupied by a metal that conferred little or no activity.

In vitro, the active site of Tdh can bind various divalent transition metals, including Fe^{2+} , Mn^{2+} , Zn^{2+} , and Co^{2+} (Anjem and Imlay, 2012). *E. coli* typically does not use cobalt, and it lacks a dedicated cobalt import system. Among the three physiologically relevant metals, Fe^{2+} confers a turnover number that is nearly 5-fold higher than those provided by Mn^{2+} or Zn^{2+} (Anjem and Imlay, 2012). The activity of Tdh extracted from the wild-type strain was sensitive to H_2O_2 (Fig. 5D), a trait that is true only of iron-cofactored enzyme, confirming that Tdh uses iron under these growth conditions. However, Tdh prepared from O_2^- -stressed cells was completely resistant to H_2O_2 (Fig. 5D), implying that the low residual activity represented Tdh that was charged with a metal other than iron. Because metals exchange during the time required to purify and characterize these enzymes, mass spectrometric metal analysis was not feasible. Instead, we identified the bound metal by characterizing the kinetic behavior of the enzyme. The prosthetic metals directly coordinate the substrates of these enzymes, and therefore the enzyme K_M is diagnostic of the metal identity. Tdh from wild type extracts had a K_M consistent with that of the Fe^{2+} -metallated enzyme, while Tdh from the SOD^- extracts exhibited a K_M perfectly matching that of the Zn^{2+} -metallated enzyme (Table 1A) (see Experimental procedures). Therefore, we concluded that Tdh is mismetallated with zinc in the O_2^- -stressed cells. In fact, the amount of residual activity matched what would be expected were the entire enzyme population loaded with zinc. Similarly, Rpe was also mismetallated with zinc in the SOD^- strain (Table 1B). The failure of the pentose-phosphate pathway (Fig. 2B) is a predictable result, since zinc-loaded Rpe is ca. 5% as active as the iron-loaded enzyme.

These results indicate that after O_2^- oxidatively removes iron from the enzymes, the resultant apoprotein is competent either to quickly re-bind iron or to inappropriately bind zinc. Over repeated cycles of iron extraction, the population becomes progressively mismetallated with zinc, and the collective activity dwindles.

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

E. coli continuously repairs oxidatively damaged [4Fe-4S] cluster dehydratases through reduction and remetallation of the residual [3Fe-4S] clusters (Gardner and Fridovich, 1992; Keyer and Imlay, 1997). The analogous reactivation of zinc-loaded mononuclear enzymes is a challenge, since the spontaneous dissociation of zinc from these proteins is extremely slow ($t_{1/2} > 8$ hours) (Sobota and Imlay, 2011). We tested whether *E. coli* has any mechanism to speed this process.

SOD⁻ strains were cultured in anaerobic medium. Under this condition, Rpe was metallated with Fe²⁺, and its activity was set as 100% (Fig. 6A). *De novo* protein synthesis was then stopped, and aeration was started. After 3 hours of aeration, Rpe had lost ~95% of its activity. The residual activity was resistant to H₂O₂⁻ treatment and exhibited a K_M value that is characteristic of Zn²⁺-metallated Rpe. The cells were then collected by centrifugation, resuspended in anaerobic medium, and incubated anaerobically at 37 °C. Despite the absence of new protein synthesis, Rpe regained all of its activity within 60 min of the return to anaerobiosis, and it was found to be fully metallated with Fe²⁺ again (Fig. 6A). Similarly, Tdh completely restored its activity within 45 min of return to the anaerobic environment (Fig. 6B). It is interesting to note that the time frames for the repair of mononuclear iron enzymes ($t_{1/2} \sim 10$ –20 min) and [4Fe-4S] cluster dehydratases ($t_{1/2} \sim 5$ min) (Gardner and Fridovich, 1992; Keyer and Imlay, 1997) are similar.

These results implied that *E. coli* contains components that actively pull zinc out of the enzyme. We observed that the sulfur-based chelated penicillamine is capable of extracting zinc from Rpe, whereas carboxylate-based chelators such as EDTA could not. Penicillamine is a cysteine analogue (Fig. S3A); both compounds contain linked sulfhydryl and amino groups that are likely to collaborate in metal binding. The intracellular concentration of cysteine has been reported to be ~200–300 μM (Park and Imlay, 2003). In fact, that amount of cysteine was sufficient to chelate zinc from purified Rpe within an hour (Fig. S3B). In contrast, glutathione, which lacks the thiol-proximal primary amino group, failed to strip zinc from the enzyme even at its much-higher physiological concentration (5 mM). Therefore, cysteine could plausibly mediate zinc extraction from mismetallated mononuclear enzymes *in vivo*. Redoxin-type proteins with extended thiolate domains, such as glutaredoxin and thioredoxin, could potentially do so as well.

In the absence of O₂⁻, overload of zinc poisons TDH by facilitating mismetallation of the enzyme in vivo

These results lead to the model that zinc competes for the metallation of the apoprotein forms of mononuclear iron enzymes (Fig. 7). The data indicate that iron normally wins this competition, but that zinc succeeds a minor fraction of the time. If this model were true, it would be expected that excessive intracellular zinc would promote mismetallation of newly synthesized protein, even in the absence of oxidative stress. The intracellular concentration of zinc is mainly held in check by the Zn(II) efflux pump ZntA (Rensing *et al.*, 1997). When wild type and the *zntA*-deficient strains were grown in medium without zinc supplementation, Tdh in both strains was metallated with iron (Fig. S4). However, when 150 μM ZnCl₂ was added to the growth medium, Tdh in the *zntA*-deficient strain was mischarged with zinc (Fig. 8). As expected, mismetallation during O₂⁻ stress was more rapid in the *zntA* mutants (Fig. S5). This result supports the notion that the role of O₂⁻ in mismetallation is simply to provide zinc with multiple opportunities to bind in the metal site, by repeatedly displacing iron. It also indicates that zinc homeostatic systems are important for preserving the function of the mononuclear iron proteins even in the absence of oxidative stress.

Discussion

A new class of O_2^- target

The reactivity of O_2^- is sharply constrained. Although formally O_2^- is a strong thermodynamic oxidant ($E_o' = +0.94$ V), its negative charge ensures that it is not attracted to electron-dense centers, and it must be protonated before it can accept an electron. The latter requirement defuses most of its reactivity, because its pK_a (4.8) is low enough to ensure that it is rarely protonated in physiological environments. Accessible metal centers constitute a rare exception: their positive charge drives O_2^- to form an electrostatic complex with the metal, and even momentary protonation then enables electron transfer. This explains the exceptional capacity of O_2^- to oxidize both iron-sulfur clusters and mononuclear iron centers. Notably, the metal centers must be solvent-accessible so that O_2^- can bind directly. Both cluster-dependent dehydratases and non-redox mononuclear enzymes meet this criterion, since the metals must be sufficiently exposed to coordinate their substrates.

This study has identified three mononuclear iron enzymes that O_2^- inactivates, and it provides evidence that the Fur transcription factor is a fourth. The actual number of such enzymes in *E. coli* is not known. Rpe and Tdh were once thought to be zinc enzymes, since after purification they were found to have associated zinc (Akana *et al.*, 2006; Johnson *et al.*, 1998), and it is likely that other supposed zinc or manganese enzymes actually employ iron in vivo. At least 100 enzymes in *E. coli* are recognized as being activated by zinc, manganese, or cobalt (<http://www.Ecocyc.org/>). The latter two metals are not routinely imported into *E. coli* (Anjem *et al.*, 2009). In general, iron is underrecognized as a divalent metal cofactor because it is rapidly oxidized in the aerobic buffers in which the metal specificity of most enzymes is tested. This problem can be circumvented by working anaerobically or by including a good reductant, such as ascorbate or cysteine. In any case, the mononuclear enzyme family greatly expands the physiological impact of O_2^- stress in *E. coli* beyond its five iron-sulfur dehydratase activities.

Does O_2^- cause any damage in SOD-proficient cells?

The calculations presented in this paper predict that endogenous O_2^- will damage mononuclear iron enzymes at a moderate rate even in SOD proficient cells. Indeed, a previous study reported that more than a two-fold diminution of SOD activity caused enzyme problems and growth defects (Gort and Imlay, 1998). Analogous calculations show that H_2O_2 concentrations are similarly held just barely beneath a concentration at which overt phenotypes appear (Seaver and Imlay, 2001). Because *E. coli* is poised at the edge of oxidative toxicity, any increase in intracellular O_2^- or H_2O_2 will push it over the edge. This vulnerability has sparked the evolution of antagonistic mechanisms in which eukaryotic hosts or competitive microbes strive to poison other bacteria by imposing oxidative stress upon them. In the best-known cases, mammals, plants, and amoebae employ an NADPH oxidase to dowse invading bacteria with H_2O_2 and HOCl. Extracellular O_2^- cannot penetrate membranes into the cytoplasm, where the vulnerable enzymes are (Craig and Schlauch, 2009; Korshunov and Imlay, 2002; Lynch and Fridovich, 1978); however, both plants and bacteria have worked around this problem by releasing redox-active quinones and phenazines (Paiva *et al.*, 2003; Inbaraj and Chignell, 2004). These compounds penetrate into competitors and catalyze electron transfer from redox enzymes to molecular oxygen, thereby generating enough O_2^- to inactivate enzymes and paralyze metabolism.

To defend themselves against such assaults, targeted bacteria sense H_2O_2 and redox compounds through their OxyR and SoxR transcription factors, respectively (Zheng *et al.*, 2001; Pomposiello *et al.*, 2001). Both factors trigger the induction of scavenging enzymes,

but a number of other defensive systems are also engaged. To specifically defend mononuclear enzymes from H_2O_2 , the OxyR regulon induces Dps, a ferritin-like protein that sequesters iron, and MntH, a dedicated manganese importer (Altuvia *et al.*, 1994; Kehres *et al.*, 2002). These proteins act in concert to replace iron with manganese in the mononuclear enzymes, an adjustment that preserves substantial activity while avoiding active-site Fenton reactions that could otherwise damage key residues (Anjem *et al.*, 2009; Anjem and Imlay, 2012; Sobota and Imlay, 2011). Interestingly, the SoxRS system does not include either Dps or MntH. This study shows that the immediate effect of O_2^- is merely to demetallate mononuclear enzymes, without producing any covalent damage; thus, sequestration of iron by Dps would exacerbate rather than fix problems, by enabling zinc to compete more successfully. The failure of SoxRS to induce MntH is less easily explained. One might expect manganese import to suppress mismetallation by zinc, and in fact extreme manganese supplementation that bypasses the need for MntH does improve the growth of SOD mutants (Al-Maghrebi *et al.*, 2002). We note, however, that SoxRS is activated by redox-cycling compounds per se rather than by O_2^- , and since these drugs have superoxide-independent toxic effects, it may be a mistake to interpret the composition of the regulon solely in terms of defense against O_2^- stress (Gu and Imlay, 2011).

Why does *E. coli* use iron rather than other metals in these enzymes?

The first two billion years of life on Earth occurred in an anoxic atmosphere. In this reducing environment iron was highly bioavailable in its soluble ferrous form, while softer metals were locked into insoluble sulfide minerals (Anbar, 2008). Iron was therefore recruited as an enzyme cofactor. It is an excellent catalyst of surface chemistry, because it requires little activation energy to transition from four- to six-coordinate geometries, a step that is integral to the binding, activation, and release of enzymatic substrates. When oxygenic photosynthesis aerated the seas, a crisis ensued: iron availability declined, and reactive oxygen species were formed. To meet this circumstance, microbes evolved eclectic iron acquisition strategies and antioxidant defenses. A secondary effect of the newly aerobic atmosphere was that zinc became much more available, with concentrations rising by perhaps six orders of magnitude (Anbar, 2008). This posed a problem: zinc can bind at divalent-metal sites, but it is much more sluggish than iron at shifting its coordination sphere. Thus, although zinc can stabilize oxyanions, it seems to be a poor substitute for iron in most *E. coli* mononuclear enzymes. In fact, the higher affinity of zinc for four-coordinate geometries probably underlies the ironic fact that it binds more tightly to these enzymes than does iron, their proper cofactor (Sobota and Imlay, 2011). To avoid the poisoning of iron enzymes by zinc, homeostatic devices arose, including the ZntA zinc export system (Rensing *et al.*, 1997) and the ZntR zinc-sensing protein (Brocklehurst *et al.*, 1999) that regulates it.

E. coli has been a key model organism for studies of oxidative stress because it is genetically accessible, its physiology is well-understood, and its ability to grow without oxygen allows oxygen-shift experiments. To date most of the findings from this bacterium have subsequently translated to other organisms, including both lower and higher eukarya. Still, it is worth noting that *E. coli* spends most of its lifetime in the anoxic gut where iron is reasonably abundant, and so its reliance upon iron as a metal cofactor is not a fatal flaw. However, committed aerobes live in iron-poor, zinc-rich environments. It will be interesting to see whether they have refined mononuclear enzyme structures to enable zinc to be a more facile surface catalyst.

Experimental procedures

Chemicals and strains

L-amino acids, ascorbic acid, antibiotics, *o*-nitrophenyl- β -D-galactopyranoside, copper-zinc superoxide dismutase (from bovine erythrocytes), catalase (from bovine liver), zinc (II) chloride, diethylenetriaminepentaacetic acid (DTPA), EDTA, NADH, NAD⁺, tris (2-carboxyethyl) phosphine (TCEP), transketolase (from baker's yeast), thiamine pyrophosphate, α -glycerophosphate dehydrogenase-triosephosphate isomerase (from rabbit muscle), ferrous ammonium sulfate hexahydrate, manganese (II) chloride tetrahydrate, D-ribose 5-phosphate disodium salt, D-ribulose 5-phosphate disodium salt, and 30% H₂O₂ were from Sigma. Casein hydrolysate was from Fluka. Glycylglycine was from Acros Organics, formate dehydrogenase (*Candida boidinii*) was from Roche Applied Science, formyl-Met-Ala-Ser was from Bachem, and Tris base was from Fisher.

Strains and plasmids used in this study are listed in Table S1. The *zntA* null mutation was introduced into new strains by P1 transduction with selection for linked kanamycin resistance markers (Miller, 1972). The presence of the mutant allele was then confirmed by PCR analysis. The single-copy *lacZ* transcriptional fusion to the *fhuA* promoter region was integrated into the λ attachment site (Haldimann and Wanner, 2001). The promoter region was amplified using the forward primer 5'-

ATATGCCTGCAGCAACAGCAACCTGCTC-3' and the reverse primer 5'-TATACCGGTACCCATTGGTATATCTCTG-3', which were designed with PstI and EcoRI restriction sites. The plasmid pAH125 was modified by replacing the kanamycin-resistance cassette with a chloramphenicol-cassette flanked by FLP sites. The promoter region was inserted into pSJ501, and the resultant plasmid was confirmed by restriction analysis.

Bacterial growth

LB medium contained 10 g tryptone, 10 g NaCl, and 5 g yeast extract per liter. Glucose / amino acids medium consisted of M9 or minimal A salts plus 0.2% glucose, 0.2% casein hydrolysate, 0.5 mM tryptophan, 5 mg/mL thiamine and 1 mM MgSO₄. To stop protein synthesis, *pro*⁻ *arg*⁻ auxotrophs were centrifuged, washed with minimal A salts, and resuspended in glucose medium without proline and arginine.

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

EPR measurement of unincorporated intracellular iron

Intracellular unincorporated iron concentrations were determined through the EPR protocol described previously (Woodmansee and Imlay, 2002), with minor modifications. Overnight bacterial cultures were prepared anaerobically in glucose / amino acids M9 medium at 37°C. Each overnight culture was then diluted into 1 L of fresh medium and aerated rigorously at 37°C. Cultures were grown to an OD₆₀₀ of 0.25. For cells carrying the plasmid pRpe, 0.5 mM IPTG was added when the culture reached 0.1 OD₆₀₀, and cells were grown up to 0.25 OD₆₀₀. Cells were harvested, and cell pellets were resuspended in 8 ml of medium

prewarmed to 37 °C. One ml of 100 mM DTPA was then added to block further iron import, followed by 1 ml of 200 mM deferoxamine mesylate. Deferoxamine penetrates cells, binds unincorporated ferrous iron, and in the presence of oxygen triggers its oxidation to EPR-detectable ferric iron. The mixture was then incubated aerobically at 37°C for 15 min. After the incubation, cells were centrifuged, washed twice with 20 mM cold Tris-Cl (pH 7.4) buffer, and finally resuspended in 250 µL of cold 20 mM Tris-Cl buffer containing 15% glycerol (pH 7.4). The cell suspension was loaded into a quartz EPR tube and frozen on dry ice. The cell density of the remaining suspension was measured for final normalization.

EPR spectra were obtained under the following conditions: microwave power, 10 mW; microwave frequency, 9.05 GHz; modulation amplitude, 12.5 Gauss at 100 KHz; time constant, 0.032; and sample temperature, 15 K. Iron concentrations were determined based on iron standards. Iron standards were prepared by making serial dilutions of FeCl₃ in 20 mM Tris-Cl containing 10% glycerol and 1 mM desferrioxamine. The iron concentrations of these standard solutions were determined using ϵ_{mM} of the desferrioxamine:Fe³⁺ complex at 420 nm of 2.865 cm⁻¹. Intracellular iron concentrations were calculated by normalizing the iron measurements to intracellular volume, using the conversion that 1 ml of 1 OD bacteria collectively contains 0.52 µl of cytosol (Imlay and Fridovich, 1991a).

Protein purification

Tdh was purified under aerobic conditions as described in (Anjem and Imlay, 2012). Cell extracts were prepared from an LB culture of *E. coli* BL21 strain containing the Tdh overexpression plasmid pET21b-Tdh. The purified enzyme had 100 U/ml of enzyme activity and was > 95% pure as indicated by SDS-PAGE analysis. It was fully charged with zinc, since the purification buffers contained 0.25 mM ZnCl₂.

PDF was purified as described in (Anjem and Imlay, 2012). Cell extracts were prepared from an LB culture of *E. coli* BL21 strain containing the PDF overexpression plasmid pET21b-PDF. The purified enzyme had 150 U/ml of enzyme activity and was > 95% pure. It was fully charged with nickel, since the purification buffers contained 0.2 mM NiSO₄.

To purify Rpe, the gene *rpe* was inserted into pET15b, and the resulting pRpe-His10 was overexpressed in *E. coli* BL21 strain. The overexpressed Rpe-His10 protein was then purified using His Gravitrapp (GE Healthcare) (Sobota and Imlay, 2011). The His tag was removed after purification. Purified Rpe (> 95% pure) had a protein concentration of 1.5 mg/ml. Since EDTA was present during purification, the purified Rpe protein existed in the inactive apoform.

Enzyme assays

Assays of threonine dehydrogenase (Tdh), peptide deformylase (PDF), and ribulose 5-phosphate epimerase (Rpe) were conducted in the anaerobic chamber at 25°C. Cell crude extracts for Tdh analysis were prepared by sonication in 50 mM Tris-HCl buffer (pH 8.4) at 4°C. The lysis buffer was anaerobic and contained 5 mM threonine to stabilize the bound metal and 100 µM DTPA to avoid adventitious metal binding. Assays were performed within 5 min of cell lysis to avoid significant metal dissociation. Pure Tdh was stored in the presence of 50 µM ZnCl₂. To load Tdh with other metals, it was incubated with 10 mM EDTA at 25°C for 30 min. Over 95% of activity was lost after this treatment. The apoenzyme was then diluted 1:50 into buffer containing 350 µM of the desired metal at 25°C. Tdh was assayed under anaerobic conditions following standard methods (Boylan and Dekker, 1981), with slight modifications. A typical assay (500 µl) consisted of 50 mM Tris-HCl buffer (pH 8.4), 1 mM NAD⁺, 30 mM threonine, and the enzyme (pure or from cell

extracts) at 25°C. Tdh oxidizes threonine, with the production of NADH that can be monitored by increase in A₃₄₀.

Cell crude extracts for PDF analysis were prepared by sonication at 4°C in 50 mM HEPES buffer with 25 mM NaCl (pH 7.5) and 100 μM DTPA. Assays were performed within 5 min of cell lysis to avoid significant metal dissociation. Pure PDF was stored in the presence of 50 μM NiSO₄. To load PDF with other metals, it was incubated with 25 mM EDTA at 25°C for 45 min. PDF lost over 95% of activity after EDTA treatment. The apoenzyme was then diluted 1:50 into buffer containing 700 μM of the desired metal at 25°C. PDF was assayed under anaerobic conditions by standard method described in (Lazennec and Meinnel, 1997). A typical assay (500 μl) contained 50 mM HEPES buffer with 25 mM NaCl (pH 7.5), 10 mM NAD⁺, 1 unit of formate dehydrogenase, 1 mM formyl-Met-Ala-Ser, and the enzyme (pure or from cell extracts). PDF removes the formyl group from formyl-Met-Ala-Ser. Formate thus generated is then oxidized to CO₂ and H₂O by formate dehydrogenase, with reduction of NAD⁺ to NADH, which is monitored by increase in A₃₄₀.

Cell crude extracts for Rpe analysis were prepared by sonication at 4°C in 50 mM glycylglycine buffer (pH 8.5) containing 1 mM DTPA. Assays were performed within 5 min of cell lysis to avoid significant metal dissociation. Rpe was purified in its apoprotein form. To charge it with metals, the enzyme was diluted 1:100,000 in buffer containing 100 μM of the desired metal at 25°C. Rpe was assayed under anaerobic conditions as described in (Kiely *et al.*, 1973). A typical assay (500 μl) contained: 50 mM glycylglycine buffer (pH 8.5), 5 mM DTPA, 1 unit of α-glycerophosphate dehydrogenase, 10 units of triosephosphate isomerase, 1 mM ribose 5-phosphate, 1 mM ribulose 5-phosphate, 0.2 mM NADH, and 1 unit of transketolase. Transketolase was purchased in its apoprotein form and was incubated with 0.2 mM MgCl₂ and 2 mM thiamine pyrophosphate for over 15 min on ice before 5-fold dilution to the assay mixture. Rpe converts ribulose-5-phosphate to xylulose-5-phosphate, which was then detected in an enzyme-linked assay through NADH consumption, as a decrease in A₃₄₀.

β-galactosidase activity was assayed as described (Miller, 1972). Total protein content was determined using the Coomassie Blue dye-binding assay (Pierce). Error bars represent SD from the mean of three independent experiments.

Superoxide challenge in vitro

O₂⁻ was generated in vitro through the reaction of xanthine oxidase. In the reaction, the enzyme transfers electrons from xanthine to O₂, producing a mixture of O₂⁻ and H₂O₂ (McCord and Fridovich, 1969). To monitor the loss of enzyme activities upon O₂⁻ challenge, purified enzymes or cell crude extracts were prepared and mixed with other assay components anaerobically in assay buffers. Catalase (110 U/ml) was added to prevent interference from H₂O₂. Xanthine (100 μM) and xanthine oxidase (15 mU/ml) were then added to the mixture aerobically. To ensure efficient O₂⁻ production, the liquid was aerated by being pipetted up and down a few times along the cuvette wall. The activities of Tdh, Rpe, or PDF were then monitored on a spectrophotometer. To test the protection effects of SOD on these enzymes, 500 U/ml of SOD was added anaerobically before the O₂⁻ challenge.

Inactivation rate constant determination

A competition assay system was employed to deduce how fast O₂⁻ inactivates mononuclear iron enzymes in vitro. A series of concentrations of SOD was mixed with other assay components anaerobically. Xanthine (100 μM) and xanthine oxidase (15 mU/ml), which generated O₂⁻ in the presence of O₂, were then added to the above mixture aerobically.

Catalase (110 U/ml) was included in all assays. The loss of enzyme activity was monitored on a spectrophotometer thereafter. SOD reacts with O_2^- at a rate of $2 \times 10^9 M^{-1} s^{-1}$. By comparing the inactivation rates of mononuclear iron enzymes in the presence and absence of SOD, one can calculate how fast O_2^- reacts with these enzymes (Flint et al., 1993).

To measure how fast molecular oxygen inactivates PDF, aerobic 50 mM HEPES buffer with 25 mM NaCl (pH 7.5) was brought into the anaerobic chamber and mixed with the rest of the enzyme assay components. To obtain 5% and 17% aerobic buffer, 25 μ l and 85 μ l of the aerobic buffer was added to a 500 μ l reaction, respectively. The reactions were sealed in anaerobic cuvettes and were not exposed to air during the assay processes. In the case of the treatment with saturated concentration of O_2 (260 μ M), O_2 was introduced by pipetting along the cuvette wall aerobically. Catalase and SOD were included in all assays.

Metallation and demetallation of enzymes

To chelate metals from the active site of Tdh, purified Tdh protein or cell extracts were incubated with 2.5 mM EDTA for 10 min at 25°C anaerobically. Tdh activity was completely gone after chelation. In the case of Rpe, 5 mM penicillamine was used instead of EDTA, and the mixture was incubated at 25°C for 40 min anaerobically.

To remetallate the enzymes, $Fe(NH_4)_2(SO_4)_2$ or $ZnCl_2$ was added to a final concentration that was 500 μ M in excess of the residual chelator concentration, and the mixture was incubated anaerobically for 8 min at 25°C to allow remetallation to take place.

Metal content determination

We were able to determine which metal occupies the active site of a mononuclear enzyme, due to the different K_M values corresponding to different metalloforms of the enzyme. Two substrate concentrations were selected: 2 mM and 150 mM of threonine for Tdh, and 0.1 mM and 4 mM of ribulose-5-phosphate for Rpe. The reaction rates at these two substrate concentrations were measured for purified enzymes loaded with Fe^{2+} , Mn^{2+} , and Zn^{2+} . The ratios of the two rates are characteristic for each metalloform of the enzymes. We then deduced the metal content of Tdh and Rpe prepared from cells by comparing their ratios to the standards from purified enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Endogenous O_2^- disrupts Fur metallation

(A) The Fur regulon is derepressed in SOD-deficient strains in defined medium. The wild type (wt) and SOD-deficient (SOD⁻) strains were grown aerobically in glucose/amino acid M9 medium to an OD₆₀₀ of ~0.25. Where indicated, 1 mM dipyrityl was added, and cells were aerated for an additional 30 min. Supplemental Figure S1 shows that the derepression was mediated by loss of Fur activity. **(B) Intracellular free iron levels.** Cells were cultured as described in (A). To induce Rpe expression, 0.5 mM IPTG was added to the culture at ~0.1 OD₆₀₀. EPR samples were prepared as described in “Experimental procedures”. Wild type, AB1157; SOD⁻, PN134; *fur*⁻, KK210; and SOD⁻ *fur*⁻, KK216.

Fig. 2. (A) Mononuclear iron enzymes are damaged in the SOD-deficient strains

The wild type (wt) and SOD-deficient (SOD⁻) strains were grown aerobically in glucose/ amino acid minimal A medium to an OD₆₀₀ of ~0.3, and enzyme activities were measured.

(B) The pentose phosphate pathway fails in SOD⁻ mutants. Cells were precultured anaerobically and then diluted into aerobic gluconate/amino acid minimal A medium. Cell growth was monitored thereafter. The Δedd mutants lack 6-phosphogluconate dehydratase and therefore depend exclusively upon the pentose-phosphate pathway to catabolize gluconate.

Fig. 3. Superoxide rapidly inactivates mononuclear iron enzymes *in vitro*

Purified Tdh (A), Rpe (B) and PDF (C) were metallated with Fe(II) anaerobically. O_2^- was generated *in vitro* aerobically by xanthine and xanthine oxidase. Where indicated, 500 U/ml of SOD was added before exposure to O_2^- .

Fig. 4. Addition of Fe²⁺ fully restores activity to O₂⁻-damaged Tdh (A) and Rpe (B)
Iron-charged enzymes were treated with O₂⁻ for 3 min in the presence of catalase, and 500 U/ml of SOD was then added to scavenge residual O₂⁻. Where indicated, 0.5 mM Fe(NH₄)₂(SO₄)₂ was then added to reactivate the enzymes, and 0.5 mM ascorbic acid was also included to keep iron in its ferrous form in the aerobic environment.

Fig. 5. O₂⁻ damages Tdh *in vivo* by causing mismetallation

(A) Tdh progressively loses activity in the SOD⁻ strain. Wild type (wt) and SOD⁻ mutant were grown anaerobically to an OD₆₀₀ of 0.2, *de novo* protein synthesis was stopped, and cultures were shifted to aerobic conditions. At indicated time points, aliquots were taken to measure Tdh activity. **(B) Tdh does not accumulate in the apoprotein form in O₂⁻ -stressed cells.** Cell extracts were prepared from SOD⁻ strain which had been aerated for 120 min. Where indicated, 0.5 mM of Fe(NH₄)₂(SO₄)₂ was added to the extracts. **(C) Tdh protein polypeptide is not degraded in O₂⁻-stressed cells.** Cell extracts were prepared from wild type and SOD⁻ strain which had been aerated for 120 min. The extracts were incubated anaerobically with 2.5 mM EDTA at RT for 10 min. The enzyme was then charged with Fe(NH₄)₂(SO₄)₂ under anaerobic conditions prior to assay (Experimental Procedures). **(D) Tdh from O₂⁻ -stressed cells is not metallated with iron.** The cell extracts from part (C) were treated with 50 μM H₂O₂ anaerobically.

Fig. 6. Rpe (A) and Tdh (B) restore activity *in vivo* after superoxide stress is removed

The SOD deficient strain (SOD⁻) was grown anaerobically at 37 °C to an OD₆₀₀ of ~0.20. Then *de novo* protein synthesis was stopped, and cells were shifted to aerobic conditions. After 3 hours of aeration at 37 °C, cells were returned to anaerobic conditions. At the subsequent time points, aliquots were harvested for measurement of Rpe and Tdh activity.

Fig. 7. Model for *in vivo* inactivation of mononuclear iron enzymes by O_2^- .

Fig. 8. Even in the absence of O_2^- stress, excess intracellular zinc causes Tdh mismetallation *in vivo*

The wild type strain (wt) and the zinc efflux deficient mutant ($\Delta zntA$) were grown in glucose/amino acid medium supplemented with 150 μM $ZnCl_2$. To test the $H_2O_2^-$ sensitivity of Tdh, 50 μM H_2O_2 was incubated with the crude extracts at RT for 3 min. To chelate metals from Tdh active site, the crude extracts were anaerobically incubated with 2.5 mM EDTA at RT for 10 min. The enzyme was then charged with $Fe(NH_4)_2(SO_4)_2$ or $ZnCl_2$ prior to assay.

Table 1

Mononuclear iron enzymes Tdh (A) and Rpe (B) are mismetallated with zinc in O₂⁻-stressed cells.

(A)		
Tdh sample	K_M (mM) ^(a)	V_{2 mM}/V_{150 mM}
Purified Tdh		
Fe-Tdh	6.5	0.25
Mn-Tdh	123	0.03
Zn-Tdh	2.8	0.42
Tdh from cell extracts		
wt		0.24
SOD ⁻		0.43
(B)		
Rpe sample	K_M (mM) ^(b)	V_{0.1 mM}/V_{4 mM}
Purified Rpe		
Fe-Rpe	1.6	0.082
Mn-Rpe	1.8	0.076
Zn-Rpe	4.8	0.044
Rpe from cell extracts		
wt		0.088
SOD ⁻		0.045

The K_M values of Tdh for Threonine ^(a) and of Rpe for ribulose-5-phosphate ^(b) are obtained from previous publications (Anjem and Imlay, 2012; Sobota and Imlay, 2011).