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Hydrogen peroxide inactivates the *Escherichia coli* Isc iron-sulfur assembly system, and OxyR induces the Suf system to compensate

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Abstract

Environmental H₂O₂ creates several injuries in *Escherichia coli*, including the oxidative conversion of dehydratase [4Fe-4S] clusters to an inactive [3Fe-4S] form. To protect itself, H₂O₂-stressed *E. coli* activates the OxyR regulon. This regulon includes the *suf* operon, which encodes an alternative to the housekeeping Isc iron-sulfur-cluster assembly system. Previously studied [3Fe-4S] clusters are repaired by an Isc/Suf-independent pathway, so the rationale for Suf induction was not obvious. Using strains that cannot scavenge H₂O₂, we imposed chronic low-grade stress and found that *suf* mutants could not maintain the activity of isopropylmalate isomerase, a key iron-sulfur dehydratase. Experiments showed that its damaged cluster was degraded in vivo beyond the [3Fe-4S] state, presumably to an apoprotein form, and thus required a de novo assembly system for reactivation. Surprisingly, sub-micromolar H₂O₂ poisoned the Isc machinery, thereby creating a requirement for Suf both to repair the isomerase and to activate nascent Fe-S enzymes in general. The IscS and IscA components of the Isc system are H₂O₂-resistant, suggesting that oxidants disrupt Isc by oxidizing clusters as they are assembled on or transferred from the IscU scaffold. Consistent with these results, organisms that are routinely exposed to oxidants rely upon Suf rather than Isc for cluster assembly.

Keywords

Iron-sulfur clusters; the Suf system; the Isc system; and oxidative stress

Introduction

Contemporary organisms inherited their biochemical mechanisms and metabolic pathways from ancestors that evolved in an anaerobic world. Problems arise when this machinery is employed in aerobic habitats. Inside cells, molecular oxygen steals electrons from redox enzymes and continuously generates superoxide and hydrogen peroxide; these oxygen species are reactive and, therefore, potentially toxic. Organisms that live in aerobic habitats protect themselves from endogenous H₂O₂ by scavenging it with catalases and peroxidases. The titers of these enzymes are high enough to drive steady-state levels of H₂O₂ to levels as low as 20 nM (Seaver & Imlay, 2001b).

Nevertheless, because H₂O₂ can cross membranes, microbes are rapidly toxified if the extracellular concentration of H₂O₂ is high. This situation can result from abiotic processes, such as the oxidation of environmental sulfur or metals. It also occurs when organisms attempt to poison competitors by exploiting their vulnerability to reactive oxygen species.

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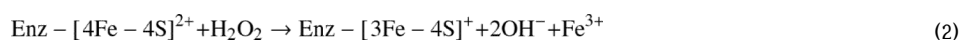
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For example, lactic acid bacteria excrete H₂O₂ that inhibits the growth of other microbes; many plants and bacteria secrete redox-cycling antibiotics, which enter competitors and generate inhibitory oxidants; and mammalian macrophages deploy NADPH oxidase, which blasts captive bacteria with superoxide and H₂O₂ (Imlay, 2008a).

To understand oxidative stress we must identify the target biomolecules that are most vulnerable to it. Early work showed that millimolar doses of extracellular H₂O₂ are moderately bacteriocidal, while micromolar doses are bacteriostatic (Imlay & Linn, 1986). The identification of the damaged biomolecules was thwarted by the robust scavenging activities of cells, which quickly degrade the H₂O₂ that is added to lab media and thus interfere with the imposition of chronic micromolar H₂O₂ stress. To circumvent this problem, one can analyze the injuries that accumulate in mutant strains that are devoid of scavenging enzymes. A mutant strain of *E. coli* that lacks both its catalases and its NADH peroxidase (*katE katG ahpCF*, denoted Hpx⁻) does not degrade H₂O₂ at a significant rate (Seaver & Imlay, 2001a). When this strain is transferred from anaerobic to aerobic medium, endogenous H₂O₂ accumulates up to 1 micromolar. Since the H₂O₂ equilibrates between the intracellular and extracellular environments, external measurements allow intracellular concentrations to be tracked. Experiments with this strain have revealed several types of cell damage that occur rapidly. The Fenton reaction between H₂O₂ and unincorporated intracellular iron [reaction 1] generates hydroxyl radicals, which oxidize a variety of biomolecules but exert their greatest toxicity by damaging DNA (Park *et al.*, 2005, Henle *et al.*, 1999, Imlay *et al.*, 1988, Levin *et al.*, 1982).



H₂O₂ also impedes the function of the Fur regulatory protein, apparently by oxidizing its iron cofactor; the consequence is a disruption of iron homeostatic mechanisms (Varghese *et al.*, 2007). H₂O₂ creates covalent polypeptide damage by reacting with the iron cofactors of metalloproteins (Anjem *et al.*, 2009). Finally, H₂O₂ oxidizes the solvent-exposed [4Fe-4S]²⁺ clusters of a family of dehydratases (Jang & Imlay, 2007, Flint *et al.*, 1993).



The catalytic iron atom is released, thereby obviating enzyme activity. A residual [3Fe-4S]⁺ cluster remains, and it is subsequently reactivated to the [4Fe-4S]²⁺ form inside cells, through a process that has not been resolved. It is striking that all these mechanisms of cell damage derive from reactions between H₂O₂ and iron.

These are the targets that cells must protect when they enter H₂O₂-containing environments. To do so, a H₂O₂-responsive regulon is activated. In *E. coli* this regulon is controlled by the OxyR protein, which responds to as little as 0.2 micromolar intracellular H₂O₂ (Carmel-Harel & Storz, 2000, Zheng *et al.*, 2001). This value appears to define a threshold concentration of H₂O₂ that is potentially toxic.

Genes that respond to OxyR should, therefore, comprise key defenses against H₂O₂; indeed, although Hpx⁻ mutants can grow in aerobic medium, Hpx⁻ *oxyR* mutants cannot (Park *et al.*, 2005). Some of the OxyR-controlled defenses have been identified. Catalase (encoded by *katG*) and NADH peroxidase (*ahpCF*) are induced in order to drive down the level of H₂O₂ (Seaver & Imlay, 2001a). Dps, a ferritin-like protein, is induced to sequester loose iron; by doing so, it greatly suppresses Fenton-mediated damage to DNA and to proteins

(Park et al., 2005, Grant *et al.*, 1998, Ilari *et al.*, 2002). Fur is induced in order to restore repression of iron-import proteins (Varghese et al., 2007). A manganese importer (*mntH*) protects simple metalloproteins by replacing their iron cofactors with an alternative metal that will not react with H₂O₂ (Anjem et al., 2009).

The *suf* operon is also induced (Zheng et al., 2001). The SufABCDSE proteins comprise an iron-sulfur-cluster assembly system. Under routine growth conditions, the Suf system is not synthesized in *E. coli*, as clusters are assembled by the housekeeping Isc system (Takahashi & Nakamura, 1999, Barras *et al.*, 2005, Bandyopadhyay *et al.*, 2008). Therefore, it seemed plausible that the Suf system is induced during H₂O₂ stress in order to repair damaged iron-sulfur clusters, in distinction to their de novo synthesis by the Isc apparatus. Consistent with this notion, experiments in some bacteria (but not others) (Tokumoto *et al.*, 2004, Runyen-Janecky *et al.*, 2008) indicated that Suf enhanced their resistance to a bolus of millimolar H₂O₂. Most strikingly, mutants of *Erwinia chrysanthemi* that lacked the *suf* operon exhibited hypersensitivity to paraquat, a generator of intracellular superoxide and H₂O₂ (Nachin *et al.*, 2001). Subsequent work showed that the activities of oxidant-sensitive dehydratases were reduced (Nachin *et al.*, 2003).

The availability of Hpx⁻ mutants enabled us to test whether Suf is important in protecting *E. coli* from physiological, sub-micromolar levels of H₂O₂. Further, it allowed us to address the question of why Isc alone is inadequate. We considered several possibilities: that Suf is designed specifically to repair damaged clusters; that Suf is dedicated to cluster assembly for a protein(s) that is exclusively used during H₂O₂ stress; or that Isc is inoperative in H₂O₂-stressed cells. We determined that sub-micromolar H₂O₂ disables the Isc system and that Suf assumes the role of both de novo synthesis and cluster repair.

Results

The Suf system is critical during H₂O₂ stress

Previous studies have shown that when *E. coli* is grown in standard lab media the Isc system constitutes the major mechanism of [Fe-S] cluster assembly (Barras et al., 2005, Bandyopadhyay et al., 2008). In agreement with that conclusion, we observed that $\Delta hscA$ mutants grew substantially more slowly in a minimal glucose medium ($t_D = 89'$) than did the wild-type parent strain ($t_D = 69'$). Moreover, the enzymatic activities of two [Fe-S]-dependent enzymes, isopropylmalate isomerase and NADH dehydrogenase I, were reduced to 25% and 10% of their wild-type values (Fig. S1). (For this purpose $\Delta hscA$ mutants were studied because they lack Isc function but retain IscS-dependent activities that are unrelated to cluster assembly.) The residual activities of these enzymes were apparently due to the action of the Suf system, which is induced in Δisc mutants by the apo-IscR transcription factor. Double Δisc *suf* mutants cannot build clusters at all and are inviable (Takahashi & Tokumoto, 2002).

In contrast, deletions of the *suf* operon caused no growth defect ($t_D = 69'$), nor were enzyme activities detectably diminished. Thus, as others have inferred (Outten *et al.*, 2004, Djaman *et al.*, 2004), the Suf system has no apparent role under standard growth conditions.

The observation that *suf* is controlled by the OxyR (Zheng et al., 2001, Lee *et al.*, 2004) protein prompted us to examine whether the Suf system becomes important when cells are exposed to H₂O₂. Other workers have noted recovery defects when Δsuf mutants were challenged with a bolus of millimolar H₂O₂ (Nachin et al., 2001, Nachin et al., 2003, Tokumoto et al., 2004). Because the OxyR system is activated by sub-micromolar concentrations of intracellular H₂O₂ (Aslund *et al.*, 1999), we sought to test the role of Suf when cells were chronically exposed to low, physiological doses. To do so we employed

Δahp katG katE (Hpx^-) mutants, which lack catalase and peroxidase activities and therefore cannot scavenge H_2O_2 . When these mutants are cultured in aerobic medium, they accumulate 0.5–1.0 micromolar H_2O_2 , which is generated by the spontaneous oxidation of intracellular redox enzymes (Seaver & Imlay, 2001b). This H_2O_2 comprises a constant oxidative stress. Hpx^- mutants are able to grow under these conditions, but they display reduced activities of some [Fe-S] dehydratases because H_2O_2 oxidatively damages their solvent-exposed [Fe-S] clusters (Jang & Imlay, 2007).

When anaerobic cultures of Hpx^- mutants were diluted into aerobic medium, the *suf* operon was expressed at a level 40-fold higher than in wild-type cells, consistent with its induction by the OxyR protein (Fig. S2). Induction was ten-fold lower when the OxyR binding site was deleted. $\text{Hpx}^- \Delta\text{suf}$ mutants grew somewhat more slowly than did their *suf*⁺ parent. The addition of 6 μM H_2O_2 completely abolished their growth of the $\text{Hpx}^- \Delta\text{suf}$ strain; under the same conditions the parent continued to grow well (Fig. 1A). The growth defect of the $\text{Hpx}^- \Delta\text{suf}$ mutant was eliminated when the *suf* operon was expressed from a plasmid (Fig. 1B). An $\text{Hpx}^- \text{suf}$ (*non-inducible*) mutant—which retained the intact *suf* operon but lacked the OxyR binding site upstream—exhibited a growth rate intermediate between that of the $\text{Hpx}^- \Delta\text{suf}$ mutant and the $\text{Hpx}^- \text{Suf}^+$ strain (Fig. S3). Thus the Suf system is critical for growth during H_2O_2 stress, and OxyR must induce it to achieve sufficient levels.

Interestingly, $\text{Hpx}^- \Delta\text{hscA}$ mutants were able to grow efficiently in aerobic medium, even when the exogenous H_2O_2 was added (Fig. 1A). This contrasts with the problems displayed by ΔhscA mutants in an Hpx^+ background, and it suggests that the OxyR-driven induction of Suf might fully compensate for the absence of the Isc system.

Most [Fe-S] enzymes are dispensable for growth in glucose medium, but isopropylmalate isomerase (IPMI) is necessary for the synthesis of leucine. Indeed, leucine supplementation restored the growth of the $\text{Hpx}^- \Delta\text{suf}$ mutant. IPMI is among the most sensitive of the dehydratases that can be inactivated by H_2O_2 , and measurements showed that its activity diminished rapidly in both $\text{Hpx}^- \text{Suf}^+$ and $\text{Hpx}^- \Delta\text{suf}$ mutants during their first hour of growth due to endogenous H_2O_2 (Fig. 2A). However, whereas the OxyR-mediated induction of *suf* ultimately enabled the IPMI activity in $\text{Hpx}^- \text{Suf}^+$ cells to regain about 10–15% of their original IPMI activity, the IPMI activity in $\text{Hpx}^- \Delta\text{suf}$ mutants did not recover. After four hours of aeration, the latter strain lacked detectable activity. The absence of IPMI in the Δsuf background occurred despite a compensatory induction of *leuCD* transcription, which is responsive to the intracellular concentration of leucine (Fig. 2B).

Fumarase A and 6-phosphogluconate dehydratase are also [Fe-S] dehydratases whose activities are vulnerable to H_2O_2 (Jang & Imlay, 2007). The activities of these enzymes were similarly lower in $\text{Hpx}^- \Delta\text{suf}$ mutants than in $\text{Hpx}^- \text{Suf}^+$ cells (Fig. 3). Therefore, we concluded that the Suf system is needed to preserve [Fe-S] dehydratase activities during low-grade H_2O_2 stress.

The Suf system is needed to repair [Fe-S] clusters that have degraded beyond the [3Fe-4S]⁺ state

In principle Suf could enhance dehydratase activity in H_2O_2 -stressed cells through either of two ways: by inserting clusters de novo into newly synthesized polypeptide, or by rebuilding clusters that had been oxidatively damaged by H_2O_2 . The preceding experiments involved growing cells and therefore did not distinguish between these possibilities; however, given the circumstances, we suspected that the Suf system facilitated cluster repair. To test this idea, we monitored the activity of dehydratases under conditions in which de novo enzyme synthesis was blocked. An $\text{Hpx}^- \Delta\text{suf}$ strain was constructed in which Suf was expressed from a plasmid under the control of the *lac* promoter; when this strain was cultured in

lactose medium, the Suf system was synthesized, even in anaerobic conditions, when it is typically not expressed. Both this strain and its $Hpx^- \Delta suf$ complement were grown to exponential phase in anaerobic lactose medium, and then chloramphenicol was added to block further protein synthesis. The cultures were then aerated, and the activity of IPMI was monitored during the subsequent period of H_2O_2 stress. The data in Fig. 4A show that the Suf system significantly decreased the rate at which H_2O_2 inactivated IPMI. Thus its protective effect was consistent with a repair activity that partially countervailed the damaging effects of H_2O_2 .

The experiment was repeated with fumarase B, the [4Fe-4S] isozyme that is expressed in anaerobic cells. Since this enzyme is less sensitive to H_2O_2 , 20 μM exogenous H_2O_2 was added to the aerobic medium. In contrast to IPMI, fumarase B activity was unaffected by the presence or absence of the Suf system (Fig. 4B). This discrepancy led us to wonder whether the damaged clusters of oxidized IPMI and fumarase B might be in different forms.

When H_2O_2 initially oxidizes an enzymic [4Fe-4S]²⁺ cluster, the cluster quickly loses its catalytic iron atom and is converted to a [3Fe-4S]⁺ form. The cluster can be rapidly repaired to the original [4Fe-4S]²⁺ state by incubation in vitro with ferrous iron and dithiothreitol; full activity is restored. We have documented this behavior for both IPMI and fumarase (Jang & Imlay, 2007). However, the iron/dithiothreitol treatment is not expected to reactivate enzymes whose clusters have degraded beyond the [3Fe-4S]⁺ state—for example, to [2Fe-2S] or apo-protein forms—as sulfur atoms must be supplied to rebuild the cluster (Urbina *et al.*, 2001, Smith *et al.*, 2001). Based on this idea, we explored the status of the oxidized clusters in IPMI and fumarase B. Figure 5A shows that iron/dithiothreitol treatment completely restored the fumarase activity in extracts prepared from H_2O_2 -exposed $Hpx^- \Delta suf$ cells. Thus all the inactive fumarase enzymes were in the [3Fe-4S] form. In contrast, the same treatment restored less than 20% of the IPMI activity. Full restoration was achieved only when purified IscS and cysteine were provided as sulfur donors, indicating that the majority of clusters were degraded beyond the [3Fe-4S] form.

The observation that IPMI clusters degraded in vivo contrasted with our previous studies (Jang & Imlay, 2007), which showed that oxidation of enzyme in vitro creates a [3Fe-4S]⁺ form that is stable for at least 60 min. We reproduced that result, using EPR to confirm cluster status. Whole-cell EPR was then used to track the cluster status when IPMI-overproducing cells were exposed to H_2O_2 . Initially, a strong [3Fe-4S]⁺ signal appeared; in contrast to the in vitro experiments, however, the signal decayed within ten minutes (Fig. 5B). During this period no activity was restored, suggesting that the disappearance of the signal was due to degradation of the cluster to a lesser form. EPR analysis failed to detect [2Fe-2S] clusters, raising the prospect that the cluster was fully removed. The mechanism of intracellular IPMI cluster degradation was probed but not solved (Discussion).

These data suggested the reason that IPMI, but not fumarase, required the Suf system for cluster repair. The Isc and Suf systems assemble full clusters on scaffold proteins, and they would therefore seem to be capable of transferring full clusters to client apoproteins; however, they would appear to be unsuitable for the reactivation of a protein that retains a partial cluster. In agreement with this idea, a recent study showed that the Isc system can reactivate apo-FNR (Mettert *et al.*, 2008). We also observed that over-expression of the *isc* operon from a plasmid diminished the rate of IPMI inactivation by H_2O_2 (data not shown), suggesting that the Suf and Isc systems work in equivalent ways, albeit the Suf system might be more effective during H_2O_2 stress.

In contrast, the reactivation of enzymes that retain [3Fe-4S] clusters appears not to require a scaffold system. When purified fumarase A was exposed to H_2O_2 in vitro, the resultant

[3Fe-4S]⁺ form of the enzyme was efficiently reactivated upon addition to cell extracts, whether or not they contained Suf proteins (data not shown). Fumarase was also repaired in vivo without any requirement for the Suf system (Fig. 6). It remains possible that some residual Isc function was sufficient to repair these mostly intact clusters, but earlier studies indicated that Isc was expendable for [3Fe-4S]⁺ repair under conditions in which Suf was poorly expressed (Djaman *et al.*, 2004). The mechanism of reactivation presumably requires the consecutive donation of an electron donor and ferrous iron, but we have not identified any cell proteins that are essential. The di-iron protein YtfE has been suggested to be a possible partner in this process (Justino *et al.*, 2006, Justino *et al.*, 2007), but under our experimental conditions the rate of IPMI inactivation was not different in Hpx⁻ and Hpx⁻ Δ ytfE mutants, and there was no difference in the rates at which [3Fe-4S]⁺ clusters of fumarase A were repaired in vivo after brief exposure to H₂O₂ (data not shown).

In summary, the Suf system is necessary for the repair of IPMI, because the oxidized IPMI cluster substantially degrades in vivo. In contrast, the [3Fe-4S]⁺ form of the oxidized fumarase cluster is stable and can be repaired without the involvement of Suf.

In H₂O₂-stressed cells the Suf system is also required for de novo cluster synthesis

Since the preceding data indicate that Suf is needed to build clusters in apo-IPMI, one might infer that the Isc system is poorly functional when *E. coli* is stressed by H₂O₂. To test this idea more directly, we examined the activation of enzymes which rely on [Fe-S] clusters but whose clusters are naturally shielded from H₂O₂ and therefore do not require repair. NADH dehydrogenase I and succinate dehydrogenase contain nine and three iron-sulfur clusters, respectively (Ohnishi, 1998, Condon *et al.*, 1985). The clusters are buried in polypeptide, and so the enzymes do not lose activity when they are exposed to even 1 mM H₂O₂ in vivo or in vitro (data not shown). These respiratory enzymes are repressed in our anaerobic glucose medium, due to the action of ArcAB and Fnr (Gunsalus & Park, 1994, Crack *et al.*, 2008), but they are induced when *E. coli* cells are aerated (data not shown). Figure 7 demonstrates that the enzyme activities quickly rose when Hpx⁻ Suf⁺ cells were transferred to aerobic medium, but not when Hpx⁻ Δ suf mutants were transferred, although there was no difference in the *nuo* transcription level between two strains. In addition, the defect in the latter cells was not due to a general problem with protein synthesis, since these enzymes are not requisite for growth in glucose medium and the Δ suf strain grew as well as its Suf⁺ partner. Instead, the result indicates that the submicromolar (0.5–1 μ M) H₂O₂ that accumulated inside cells during these experiments was sufficient to prevent the Isc system from inserting new clusters into nascent polypeptides. In contrast, the progressive induction of the Suf system by OxyR circumvented this block.

To double-check this result, we indirectly monitored the presence of [2Fe-2S] clusters in the IscR protein. When cluster assembly is defective, the accumulation of apo-IscR protein allows it to serve as a transcription factor that activates expression of the *suf* operon (Giel *et al.*, 2006, Yeo *et al.*, 2006, Lee *et al.*, 2008). A *lacZ* transcriptional fusion was constructed immediately downstream of the *suf* promoter, and the upstream OxyR binding site was removed, so that expression was primarily dictated by the status of IscR. This fusion was expressed at a two-fold higher level in an Hpx⁻ strain relative to Hpx⁻ Δ iscR mutants. A further two-fold induction was detected in Hpx⁻ Δ suf mutants. These data confirm that H₂O₂ interferes with the activity of the Isc system (Fig. 8A), and they show that the Suf system substantially compensates.

The contribution of apo-IscR to *suf* induction during H₂O₂ stress was moderate (Fig. S2 and 8A) and may not be sufficient to ensure iron-sulfur assembly. Presumably the much larger impact of the OxyR system justifies the inclusion of *suf* in that regulon.

The Isc system is present but non-functional during H₂O₂ stress

Collectively, these data indicate that the Suf system is induced in H₂O₂-stressed cells because the Isc system is inactive. One explanation for its inactivity might be that its synthesis was repressed. A transcriptional fusion to the *iscSUA* promoter was constructed; the fusion retained the RyhB sRNA binding site to ensure that Fur-mediated control was active (Desnoyers *et al.*, 2009). We observed that the expression of the Isc system was very low in anaerobic wild-type cells, and it rose rapidly when the cells were aerated. The same result was observed in Hpx⁻ cells, indicating that the dysfunction of the Isc system was not due to a lack of expression (Fig. 8B). In fact *isc* expression was somewhat higher in the H₂O₂-stressed cells than in unstressed cells, presumably reflecting substantial conversion of [2Fe-2S]⁻ IscR, a repressor of *isc*, to the apo-IscR form (Giel *et al.*, 2006, Schwartz *et al.*, 2001?). Western blot analysis using anti-IscU antibody verified the presence of this Isc protein in Hpx⁻ cells (Fig. 8C). Thus H₂O₂ apparently inhibits the function of the extant Isc machinery.

The inactivity of the Isc system is reversible

We sought to identify the mechanism by which H₂O₂ disrupts the Isc system. As a first step, we tested whether the system would regain function when H₂O₂ was removed. To do so, we took advantage of the observation that rapidly synthesized [Fe-S] proteins transiently accumulate in an apo-protein form, due to the insufficient synthesis of [Fe-S] clusters. The *fumA* gene was strongly induced from a *tac* promoter in aerobically grown wild-type and Hpx⁻ Δ *suf* strains. Under these conditions ca. 80% of the protein accumulated in the apo-protein form. Chloramphenicol was added to block further fumarase and Isc synthesis. When aerobic incubation continued, the wild-type cells progressively activated fumarase; however, no significant increase in fumarase activity occurred in the Hpx⁻ Δ *suf* strain, indicating that the accumulated H₂O₂ continued to inhibit the Isc system. In contrast, when cells were moved to anaerobic medium, fumarase activity rebounded in the Hpx⁻ Δ *suf* strain at a rate comparable to that of wild-type cells (Fig. 9). Thus once H₂O₂ stress ceases, the Isc system regains activity. This reversibility may explain why *E. coli* continues to synthesize the Isc proteins even when inhibitory levels of H₂O₂ are present. It also constrains the possible mechanisms by which H₂O₂ blocks Isc function.

How does H₂O₂ poison the Isc system?

The Suf system is induced during iron starvation (Outten *et al.*, 2004), which led to the observation that the Isc system works poorly when iron levels are low. During H₂O₂ stress the OxyR system works to minimize Fenton chemistry by reducing the levels of free iron in the cell. This is accomplished by induction of the Dps ferritin-like protein, which sequesters unincorporated iron, and by induction of the Fur repressor, which diminishes synthesis of iron importers (Park *et al.*, 2005, Varghese *et al.*, 2007). Thus we explored the possibility that the effects of the OxyR-directed responses might starve the Isc system for iron.

Hpx⁻ Δ *oxyR* mutants are not viable in aerobic medium (Park *et al.*, 2005), which precluded the most direct test of this notion; however, the OxyR system is also induced in *ahpCF* mutants, due to modestly elevated levels of intracellular H₂O₂ (Seaver & Imlay, 2001b). Assays showed that NADH dehydrogenase I was synthesized at normal rates in a *ahpCF* *suf* strain (data not shown). Further, an *oxyR2* expression plasmid that constitutively induces the OxyR response (Kullik *et al.*, 1995) also did not create either growth defects or [Fe-S] enzyme deficiencies in a *suf* background (Fig. 10A). These data indicated that OxyR-controlled activities, including iron-depletion measures, are not sufficient to inactivate Isc. Indeed, EPR measurements, indicated that due to cluster damage the levels of unincorporated intracellular iron are actually higher in Hpx⁻ mutants, in which Isc fails, than in wild-type cells, in which Isc is functional (Varghese *et al.*, 2007). Finally, a cluster-

synthesis defect could only be elicited in the ΔahpF *suf* strain when it was exposed to 20 μM exogenous H_2O_2 ; this defect was not diminished by the deletion of the *dps* gene (data not shown). Thus it appears to be H_2O_2 itself that poisons Isc function.

The Isc system consists of the IscS desulfurase, the IscU scaffold protein on which nascent clusters are assembled, an IscA protein that serves either in cluster transfer or iron delivery, a ferredoxin that might adjust the cluster redox state, and the HscA/HscB chaperone proteins that apparently assist in cluster transfer to client apoproteins (Barras et al., 2005, Bandyopadhyay et al., 2008, Ayala-Castro et al., 2008). To date only a few chemical effects of low-micromolar H_2O_2 have been detected in vivo; these include the oxidation of reactive cysteinyl residues and of solvent-exposed iron-sulfur clusters (Imlay, 2008a). The Isc system involves both, and either type of reaction would be consistent with the reversibility of Isc inactivation. Thus the potential targets were examined in turn.

The IscS desulfurase features an exposed active-site cysteine (Cupp-Vickery et al., 2003) that seemed to be a plausible target for oxidation. Most protein cysteine residues react far too sluggishly to be affected by micromolar H_2O_2 , but the OxyR, AhpC, and OhrR proteins feature active-site cysteines that are activated by local context so they are easily oxidized to sulfenic acid (Imlay, 2008a). However, we observed that purified IscS retained full activity when it was exposed to even 1 mM H_2O_2 , whether the H_2O_2 was added prior to or during the desulfurase reaction (Fig. 10B).

Uniquely among Isc/Suf proteins, IscA and SufA can substitute for one another in vivo. Although ΔiscA *sufA* mutants are inviable, indicating that Fe/S biogenesis requires one or the other of these proteins, a ΔsufA mutant that lacks any of the Isc proteins other than IscA is viable, showing that the Suf system can function using IscA (Lu et al., 2008, Vinella et al., 2009). We observed that Hpx^- mutants exhibited growth defects if they additionally lacked SufBCD, but Hpx^- ΔsufA mutants were healthy (Fig. 10C). The implication is that IscA is functional in H_2O_2 -stressed cells. Thus the Isc dysfunction must arise from damage to a different Isc protein.

The overexpression of the full *iscSUA-hscAB-fdx* gene set restored robust cluster synthesis in the Hpx^- Δsuf mutants; however, overexpression of individual genes failed to do so (Fig. 10D). Ferredoxin may dissociate during the catalytic cycle; if so, the fact that ferredoxin overproduction did not restore Isc function strongly suggests that it is not the limiting component in the H_2O_2 -stressed system. Although we observed that in vitro ferredoxin was rapidly oxidized by H_2O_2 , the oxidized cluster was fully reducible, indicating that the apparent Fenton chemistry did not generate disabling damage to the protein (Fig. S4).

Aside from IscA and ferredoxin, the other *isc* gene products likely exist as part of a larger complex (Py & Barras, 2010) so that overproduction of a single member cannot easily compensate for damage. Our suspicion was that the nascent iron-sulfur clusters that are built on IscU might be vulnerable to oxidative degradation in vivo, since they are likely to be solvent-exposed when they are transferred to recipient proteins. Further, workers have reported that clusters in IscU are degraded when they are exposed to aerobic buffers in vitro (Agar et al., 2000, Shimomura et al., 2007). To test this idea in vivo, we attempted to overproduce the IscSU complex in order to use whole-cell EPR to monitor the status of IscU clusters during exposure to H_2O_2 . In agreement with a previous report, this effort was unsuccessful (Raulfs et al., 2008); we were able to visualize clusters by EPR or visible spectroscopy only when the full *iscSUA-hscAB-fdx* operon was overexpressed. When these cells were exposed to 0.01–1 mM exogenous H_2O_2 , no $[\text{3Fe-4S}]^+$ signal appeared (Fig. S5A). Treatment of the unstressed cells with dithionite generated a signal that was consistent with $[\text{2Fe-2S}]^+$ and/or $[\text{4Fe-4S}]^+$ clusters; previous treatment with as much as 1 mM H_2O_2

did not diminish the dithionite-generated signal, indicating that H₂O₂ did not destroy the clusters (Fig. S5B). However, since the EPR analysis showed the total [Fe-S] cluster spectrum of the Isc system, oxidation or degradation of the cluster on IscU might be obscured by the dominant [2Fe-2S] signals of IscA and ferredoxin. Unfortunately, we were unable to make any definitive conclusion regarding the stability of IscU-bound clusters.

Discussion

When Barras and his colleagues exposed *E. chrysanthemi suf* mutants to phenazine methosulfate, a redox-cycling drug that generates reactive oxygen species, they discovered that dehydratase activities were very low (Nachin et al., 2003). Oxidants can damage dehydratase clusters (Flint et al., 1993, Jang & Imlay, 2007), and it seemed plausible that Suf was needed to repair them. But the observation was puzzling in two respects. First, why would a scaffold system be required to repair the [3Fe-4S] clusters that are produced by oxidation reactions? And second, in any case, why was the housekeeping Isc system inadequate for the job? The results from the present study begin to answer those questions. First, although damaged clusters are stable in vitro, some [3Fe-4S] clusters are further disassembled in vivo, thereby requiring the engagement of a de novo-style cluster-assembly system for their full repair. And second, the Isc system is poisoned by as little as 1 micromolar H₂O₂, leaving the apparently oxidant-resistant Suf system as the only viable Fe/S scaffold system. Thus during periods of oxidative stress bacteria rely upon the Suf system not only to maintain dehydratase activity but to support the activities of all Fe-S enzymes (Fig. 11). Still, these results raise some intriguing questions.

How are damaged clusters disassembled in vivo?

Repair of [3Fe-4S] clusters can easily be managed in vitro by dithiothreitol and ferrous iron, and we imagine that an analogous process occurs in vivo. A dithiol is required for reactivation, which suggests that the iron-sulfur bonds in the cluster can reorganize themselves internally and can interact with solutes. This fact led us to wonder whether the degradation of the [3Fe-4S] IPMI cluster inside cells might occur through sulfur-exchange reactions with metabolites. To test this notion, we attempted to replicate degradation by oxidizing IPMI in vitro and then incubating its [3Fe-4S] form with cysteine and with both oxidized and reduced glutathione; however, the [3Fe-4S]⁺ cluster remained intact. Thus it seems likely that degradation is catalyzed by enzymes. The Suf system is not requisite, since disassembly occurs in *suf* mutants. At present the basis of IPMI cluster decay remains uncertain.

Interestingly, the [4Fe-4S] cluster of IRP-1—which controls iron levels in mammalian cells—appears to behave much like that of IPMI, with potentially important consequences for metabolism. Workers found that in vitro H₂O₂ treatment converted the IRP-1 cluster to a [3Fe-4S] form, which lacks both the aconitase activity of the holo-protein and the RNA-binding activity of the apo-protein (Brazzolotto *et al.*, 1999). However, upon oxidation in vivo the cluster was fully degraded, thereby exposing the RNA binding site that interacts with the transcripts of iron-import and -storage proteins (Castro *et al.*, 1998, Cairo & Recalcati, 2007). Accumulation of the apo-protein form is normally a marker of iron starvation; thus, during oxidative stress the generation of the apo-protein form may inadvertently activate mechanisms that import more iron into the cell—thereby potentially aggravating the situation by stimulating Fenton chemistry. Aconitases have been proposed to serve similar iron-regulatory roles in *B. subtilis* and *E. coli* (Tang et al., 2005) and further experiments will be needed to determine whether oxidative stress affects them in a manner similar to IRP-1.

How does H₂O₂ poison the Isc system?

By refuting other possibilities, we inferred that H₂O₂ probably disrupts the Isc system by oxidizing the nascent clusters that are formed on the IscU scaffold protein. It seems particularly likely that the clusters are exposed to solvent—and to dissolved oxidants—when they are transferred to recipient apoproteins. It will be a challenge to test this idea biochemically, since H₂O₂ reacts rapidly with the ferrous iron that is used as an Isc substrate during turnover; in experimental systems this Fenton reaction will both scavenge the H₂O₂ and deplete the ferrous iron. Molecular oxygen does the same thing, forcing workers to use anaerobic conditions to study Isc function. Thus, it is not instructive to demonstrate that H₂O₂ disrupts continuous cluster assembly *in vitro*. Perhaps spectroscopic methods can reveal whether H₂O₂ interferes with single-turnover reactions.

Electron transfer from iron to H₂O₂ is an inner-sphere process; that is, in order to react with iron, H₂O₂ must bind it directly. For this reason dehydratase clusters can be fully protected from H₂O₂ by bound substrates, which provide fifth and sixth ligands to the erstwhile solvent-exposed iron atom. The iron atoms in IscU clusters are comparatively undercoordinated, as each derives three ligands from bridging sulfur atoms and another either from a protein aspartate or cysteine residue (Bandyopadhyay *et al.*, 2008). Conceivably, they can be directly oxidized by species that would occupy a fifth coordination site. In fact, using UV-visible spectroscopy, other workers showed that H₂O₂ can directly oxidize [2Fe-2S] clusters even in resting IscU protein (Bitoun *et al.*, 2008). (IscU-bound [4Fe-4S] clusters were not tested.) However, in those experiments approximately millimolar H₂O₂ concentrations were used, which is a thousand times the concentration of H₂O₂ that we found was sufficient to inhibit Isc *in vivo*. Thus the physiological relevance of this mechanism of cluster oxidation is uncertain.

If Isc inactivation were to derive from cluster oxidation, it would fall in line with the other molecular injuries produced by H₂O₂, all of which arise from iron-oxidation reactions. This includes DNA damage (Park *et al.*, 2005), which is driven by a Fenton reaction between H₂O₂ and DNA-bound iron; dehydratase inactivation (Flint *et al.*, 1993, Jang & Imlay, 2007); disruption of Fur protein, in which H₂O₂ apparently oxidizes the ferrous iron cofactor (Varghese *et al.*, 2007); and protein carbonylation, which also arises through Fenton chemistry (Anjem *et al.*, 2009). To date no non-iron-related injuries have been detected when bacteria are exposed to micromolar concentrations of H₂O₂. The rate constants for these reactions depend on the coordination environment of the iron atom but approximate 10⁴ M⁻¹ s⁻¹ (Park *et al.*, 2005, Jang & Imlay, 2007). The OxyR system is calibrated appropriately, responding when H₂O₂ levels exceed 0.2 micromolar (Carmel-Harel & Storz, 2000, Imlay, 2008a)—that is, at a dose above which the half-time of Fenton reactions approaches 1 min.

Since superoxide oxidizes dehydratase iron-sulfur clusters at a rate even higher than does H₂O₂, we wondered whether superoxide might also be able to inactivate Isc. We found that a *suf::lacZ* fusion is substantially induced in SOD⁻ mutants (Fig. S6). Further, 6-phosphogluconate dehydratase and succinate dehydrogenase activities were substantially reduced when a *suf* mutation was introduced into the SOD⁻ strain. The data indicate that superoxide, like H₂O₂, inhibits Isc function—which is consistent with an iron-sulfur target in the system. Indeed, the earlier observation that *suf* mutants are poisoned by phenazine methosulfate (Nachin *et al.*, 2003) might owe more to superoxide, which is the direct product of PMS cycling, than to H₂O₂.

Does the distribution of the Suf and Isc systems correlate with environmental stress?

The fact that artificial Isc overproduction can suppress the growth defects of $Hpx^- \Delta suf$ mutants could, in principle, suggest that Suf induction simply elevates the total cellular capacity for cluster assembly during a period of high demand—rather than that the Suf machinery is intrinsically more H_2O_2 -resistant than is the Isc machinery. This notion is not excluded by any of the data in this study. However, if this were the case, one must wonder why the cell must switch from one assembly machine to the other. This consideration suggests that the Suf system somehow shields its nascent clusters from small oxidants. Similarly, the Suf system is more resistant to soft metals (Ranquet *et al.*, 2007, Macomber & Imlay, 2009), which can disrupt solvent-exposed clusters, than is the Isc system. How Suf might shelter its clusters is entirely unclear. In any case, if the Suf system is more robust than the Isc system, one might guess that Isc is maintained in the biological world because it has its virtues, too, perhaps being catalytically more efficient than Suf. This notion has not been tested, but it fits the fact that *E. coli* employs Isc as its housekeeping system and Suf only as an emergency back-up.

More generally, one might expect that organisms that encounter oxidants routinely might dispense with Isc and exclusively express Suf, whereas ones that do not encounter oxidants might do the opposite. A phylogenetic survey of the two systems gives mixed results. Lactic acid bacteria, which generate millimolar doses of H_2O_2 through their pyruvate and lactate oxidases, contain Suf and lack Isc. So do eukaryote pathogens such as *Mycobacterium tuberculosis*, *Enterococcus faecalis*, and *Xylella fastidiosa*, which need to withstand the H_2O_2 that is generated by the host immune response (Robinson, 2009, Averyanov, 2009). Chloroplasts, which are substantial sources of reactive oxygen species, employ only the Suf system (Asada, 2006). These data are consistent with the hypothesis. However, most anaerobic bacteria also have dispensed with the Isc system, relying instead on Suf or on the Nif system. Perhaps the Isc system is ill-suited for environments that are occasionally iron-limited. An alternative is that these microbes must periodically contend with the H_2O_2 that is chemically generated when aerated waters mix with anaerobic solutions of reduced sulfur and metals. Obligate anaerobes all express catalases and/or peroxidases (Imlay, 2008b), presumably for such a circumstance.

In contrast, non-photosynthetic eukaryotes are distinguished among organisms in that they rely entirely on Isc for cluster assembly—they do not have Suf even as a back-up system. This unique arrangement may be viable only because Fe-S assembly in eukaryotes occurs in the mitochondrial matrix and thus is shielded from the external environment by two membranes. Because membranes are only semi-permeable to H_2O_2 , efficient scavenging systems are able to reduce the cytoplasmic H_2O_2 concentration to a concentration that is an order of magnitude lower than that outside the cell. Mitochondria have additional scavengers (Park *et al.*, 2000, Petrova *et al.*, 2004), so that second gradient would exist between the mitochondrial matrix and the cytosol. The effects of these layers of scavenging should be multiplicative, and it should rigorously protect the Isc system from external H_2O_2 —apparently making the Suf system unnecessary. We note, though, that these diffusion barriers would not protect Isc from drugs like antimycin, which produces H_2O_2 within mitochondria (Panduri *et al.*, 2004). Such drugs potentially diminish the activities not only of oxidant-sensitive dehydratases but also of other Fe-S enzymes whose de novo activation would be impeded.

Materials and Methods

Reagents

Glycerol and D-glucose were obtained from Fisher Scientific; ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) and sodium dithionite, from Fluka; Amplex Red, from Invitrogen; His Gravitrap, from GE Healthcare; and ECLTM Western Blotting Detection Reagents, from Amersham. Sigma was the source of L-amino acids, bovine liver catalase, horse heart cytochrome c, rabbit muscle lactate dehydrogenase, chloramphenicol, ferric chloride, ferrous ammonium sulfate, 2,2'-dipyridyl, citraconate, disodium L(-) malic acid, D,L-dithiothreitol, hydrogen peroxide, isopropyl β -D-thiogalactopyranoside, β -lactose, NADH, deamino NADH (nicotinamide hypoxanthine dinucleotide, reduced form), trichloacetic acid, N, N-dimethyl-p-phenylenediamine, potassium D-gluconate, glutathione (reduced and oxidized), sodium sulfide, succinic acid, tri(cyclohexylammonium) 6-phosphogluconic acid, plumbagin, thiamine, o-nitrophenyl- β -D-galactopyranoside, DEAE-sepharose (DFF: Fast flow and DCL-6B), and horseradish peroxidase-conjugated anti-rabbit goat immunoglobulin G. Anti-*A. vinelandii* IscU antibody was a kind gift of Dr. Dennis R. Dean.

Bacterial strains

The strains and plasmids used in this study are listed in Table 1. Null mutations were created by the Red/Gam recombination method (Datsenko & Wanner, 2000) and were confirmed by either PCR analysis or enzyme assays. Mutations were introduced into new strains by P1 transduction and selected on media containing appropriate antibiotics. All strains in the Hpx⁻ background were constructed anaerobically to avoid an outgrowth of suppressors, which may occur aerobically.

Growth conditions

Anaerobic cultures were grown in an anaerobic glove box (Coy Laboratory Products Inc.), and aerobic cultures were grown with vigorous shaking in a water bath at 37°C. Standard minimal medium contained minimal A salts, 0.2% glucose, 1 mM MgCl₂, 5 mg/liter thiamine, and 0.5 mM histidine. Histidine was routinely added to the media because the parent strain, MG1655, is a histidine auxotroph under anaerobic conditions; to minimize the difference between anaerobic and aerobic cultures, histidine was also added to aerobic cultures. Casamino acids or specified L-amino acids were present at 0.2 % or 0.5 mM, respectively.

To ensure that Hpx⁻ derivatives were growing exponentially before they were aerated, anaerobic overnight cultures were diluted to OD₆₀₀ = 0.005 in appropriate fresh anaerobic media and grown to OD₆₀₀ ~ 0.1 prior to dilution into aerobic media.

Enzyme assays

For assays of labile [4Fe-4S]-cluster-containing enzymes--isopropylmalate isomerase (IPMI), fumarase, and 6-phosphogluconate dehydratase--cell extracts were prepared by sonication in an anaerobic chamber using anaerobic buffers. IPMI activity was measured by monitoring the decrease of citraconate, a pseudo-substrate, at 235 nm in 100 mM Tris-Cl (pH 7.6). The fumarase activity was determined in 50 mM sodium phosphate (pH 7.4) containing 50 mM of L-malate; production of fumarate was monitored at 250 nm (Jang & Imlay, 2007). To assay 6-phosphogluconate dehydratase, lysates were prepared from cultures grown in a minimal medium containing 0.2 % gluconate. The production of pyruvate by 6-phosphogluconate dehydratase (in 50 mM Tris-Cl, pH 7.65) was determined in the second reaction catalyzed by lactate dehydrogenase (Gardner & Fridovich, 1991).

Inverted membrane vesicles were prepared for NADH dehydrogenase I (Ndh1) and succinate dehydrogenase (Sdh) assays. The activities were measured in 50 mM aerobic potassium phosphate buffer (pH 7.8) by monitoring either deamino NADH (nicotinamide hypoxanthine dinucleotide, reduced form) oxidation by Ndh1 at 340 nm or cytochrome c reduction for Sdh at 550 nm in 50 mM aerobic potassium phosphate buffer (pH 7.8) (Djaman et al., 2004).

The desulfurase activity of IscS was assayed anaerobically by measuring sulfide production as described before with modifications (Siegel, 1965). Purified IscS was incubated with 2.5 mM cysteine and 5 mM dithiothreitol at 37°C for 5 min, and then the reaction was stopped by addition of 20 % trichloroacetic acid. Precipitated IscS was removed by centrifugation, and the supernatant was incubated with 2 mM N, N-dimethyl-p-phenylenediamine (DPD) and 3 mM FeCl₃ at ambient temperature for 30 min in the dark. The concentration of methylene blue that results from the reaction of DPD and sulfide was then measured at 670 nm.

As a reporter enzyme for transcriptional fusions, β -galactosidase was assayed using o-nitrophenyl- β -D-galactopyranoside (ONPG) as a substrate. The final product, ortho-nitrophenol, was detected at 420 nm (Miller, 1972).

H₂O₂ concentrations in culture media were determined by the Amplex Red/horseradish peroxidase method (Seaver & Imlay, 2001b). Because H₂O₂ rapidly equilibrates across the membranes of cells that lack scavenging activities, the extracellular H₂O₂ concentration is an excellent approximation of the intracellular concentration.

Plasmid constructions

The open reading frames of targeted genes were PCR-amplified from *E. coli* MG1655 by using the primers in Table 2. The PCR products were digested with XbaI and EcoRI and cloned into either pWKS30 or pCKR101 vectors. The plasmid constructions were confirmed by restriction/sequencing analyses. In addition, complementation experiments were performed in the Hpx⁺ backgrounds to verify that the plasmids were functional. The proteins encoded by pWKS30 or pCKR101 derivatives were expressed in lactose or glucose/1 mM IPTG media, respectively.

Construction of transcriptional fusions

Single-copy chromosomal transcriptional fusions were constructed by the CRIM plasmid-host system (Haldimann & Wanner, 2001). Promoter regions of genes were PCR-amplified with primers in Table 2 and inserted into pSJ501 at KpnI and PstI restriction sites. The plasmids that contain transcriptional fusions were then integrated at the *attB* site (*att_L*) on chromosomal DNA by the integrase that is provided by pAH57. Single-copy integrants were verified by PCR. The transcriptional fusions in chromosomal DNA were introduced into desired strains by P1 transduction. Unless otherwise indicated, each fusion strain retained a working wild-type copy of the gene at its normal locus.

Inactivation and reactivation of enzymes

Cells were grown anaerobically for at least four generations to OD₆₀₀ = 0.2. H₂O₂ was added to the cultures when they were aerated. At time points, aliquots were removed, catalase was added to 200 U/ml, and cells were returned to the anaerobic chamber for centrifugation, lysis, and assay.

In vivo inactivation of IPMI by endogenous H₂O₂ was initiated by aerating anaerobic Hpx⁻ cultures without any addition of exogenous H₂O₂. Under these conditions the cells steadily

generate H_2O_2 , which was measured to be 0.5–1 μM . Cell extracts were then prepared and assayed in the anaerobic chamber.

The inactivation of enzymes *in vitro* was accomplished by the addition of H_2O_2 to lysates or to purified enzyme in anaerobic buffer. The H_2O_2 was subsequently removed by catalase prior to anaerobic assay. In some cases, damaged [3Fe-4S] clusters were chemically repaired by incubation with 100 μM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ and 2.5 mM dithiothreitol (DTT) for at least 10 min at room temperature. *In vitro* reconstitution of more extensively degraded iron-sulfur clusters was carried out by incubating enzymes in 50 mM Tris-Cl (pH 7.65) containing 500 μM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, 5 mM DTT, 2.5 mM cysteine, and purified IscS (0.16 mU) at room temperature for 20–60 min.

In vivo repair of fumarase was monitored in Hpx^- cultures. Cells were incubated with 20 μM H_2O_2 in the presence of chloramphenicol for 5 min. Catalase was subsequently added to terminate H_2O_2 stress and fumarase activities were measured over time.

Purification of enzymes

Amplified *iscS* and *fdx* were inserted in pET15b at NdeI and BamHI restriction sites. *E. coli* BL21(DE3) cells containing pIsc-(His)₆ were grown aerobically in LB to OD₆₀₀ of approximately 0.1, and 1 mM IPTG was added, followed by another four-hour incubation at 37°C. The overexpressed IscS-(His)₆ was purified using His Gravitrap (GE healthcare). The activity of the purified His-tagged enzyme was 22 mU/mg.

To purify ferredoxin, the Hpx^- strain that contains pTrcfdx was grown anaerobically to OD₆₀₀ ~ 0.1 at 37°C, and 1 mM IPTG was added. To achieve the optimum synthesis of ferredoxin, the cells were cultured for another 12 hours at room temperature. The cells were then harvested by centrifugation, and the cell pellets were resuspended in 15 ml of anaerobic 50 mM Tris-Cl/10 mM β -mercaptoethanol/0.1 mM EDTA (TBME, pH 7.4). Cell lysates were prepared by sonication, and cell debris was removed by centrifugation. The purification was performed as described before with modifications (Ta & Vickery, 1992). All steps of the purification were conducted in an anaerobic chamber at room temperature, and all buffers were anaerobic. The cell extract was loaded onto a DEAE-Sepharose column (DFF: Fast flow) and eluted by 0.2 M of KCl in TBME. The fractions that showed the visible spectrum of $[\text{2Fe-2S}]^{2+}$ clusters were pooled and then loaded onto the second DEAE column (DCL-6B). The proteins were eluted by 0–0.4 M gradient of KCl in TBME. The purified ferredoxin was concentrated using Amicon ultra-15 centrifugal filter (3 kDa cutoff). The fractions that showed the spectrum of $[\text{2Fe-2S}]^{2+}$ clusters were frozen in dry ice containing ethanol. Purified ferredoxin was more than 90% pure based on SDS-PAGE and was quantified by dye-binding assay using bovine serum albumin as a standard.

Fumarase A was overproduced and purified as described previously (Jang & Imlay, 2007).

Western blot analysis

Cells were grown for at least four generations anaerobically to an OD₆₀₀ of approximately 0.1. Aerobic growth was then initiated by vigorous aeration of the cultures. At time points cells were harvested, and samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Anaerobic cultures were also harvested as controls. Samples that contained 42 μg of proteins were loaded on 12 % SDS-PAGE gel. Gels were either stained with Coomassieblue dye or transferred to nitrocellulose. After blotting with a 5% skim milk solution, a 1:1,000 dilution of rabbit serum containing anti-*A. vinelandii* IscU was used as the primary antibody. Horseradish peroxidase-conjugated anti-rabbit goat immunoglobulin G was used as the secondary antiserum (1:20,000 dilution). The

detection process was performed using chemiluminescent reagents (ECL™ Western Blotting Detection Reagents).

EPR analysis of *Isc* proteins

Hpx⁻ cells expressing genes of the *isc* operon behind the *tac* promoter were grown in minimal glucose medium anaerobically, and 1 mM IPTG was added when cells were at an OD₆₀₀ of 0.2. After another 4 hours of incubation, the cells were harvested by centrifugation at 7500 rpm for 10 min, and the cell pellets were resuspended in 1/500 of original culture volumes of 10 % glycerol. The resuspended cells were incubated with H₂O₂ for 5 min at room temperature, and then catalase was added to remove H₂O₂. In the attempts to detect [2Fe-2S] or [4Fe-4S] clusters, 10 mM sodium dithionite was added. The cell suspension (250 μl) then was transferred into an EPR tube and frozen in dry ice.

EPR spectra of [Fe-S]clusters were obtained with the following settings: microwave power, 1 milliwatt; microwave frequency, 9.05 GHz; modulation amplitude, 8 Gauss at 100 KHz; time constant, 0.032; temperature, 15 K.

Intracellular free-iron measurements

Cells were grown in minimal glucose medium to an OD₆₀₀ of 0.1–0.2 and harvested by centrifugation at 7000 g for 5 min at 4°C. A cell pellet was resuspended in 8 ml prewarmed fresh minimal medium that contain 10 mM DETAPAC (diethylenetriaminepentaacetic acid, pH 7.0) and 20 mM desferrioxamine (pH 8.0). DETAPAC blocks further iron import, while desferrioxamine diffuses into cells and binds unincorporated iron in an EPR-visible ferric form. The concentrated cells were incubated at 37 °C for 15 min shaking in a water bath. The cells were washed with 5 ml of ice cold 20 mM Tris-Cl (pH 7.4) twice. Cells were then resuspended in 200 μl of ice cold 10% glycerol/20mM Tris-Cl (pH 7.4). The cell suspension (200 μl) then was transferred into an EPR tube and frozen in dry ice. Ferric sulfate standards were mixed with desferrioxamine and prepared in the same Tris buffer containing glycerol. The spectrometer settings were as follows: microwave power, 10 milliwatt; microwave frequency, 9.05 GHz; modulation amplitude, 12.5 Gauss at 100 KHz; time constant, 0.032; temperature, 15 K.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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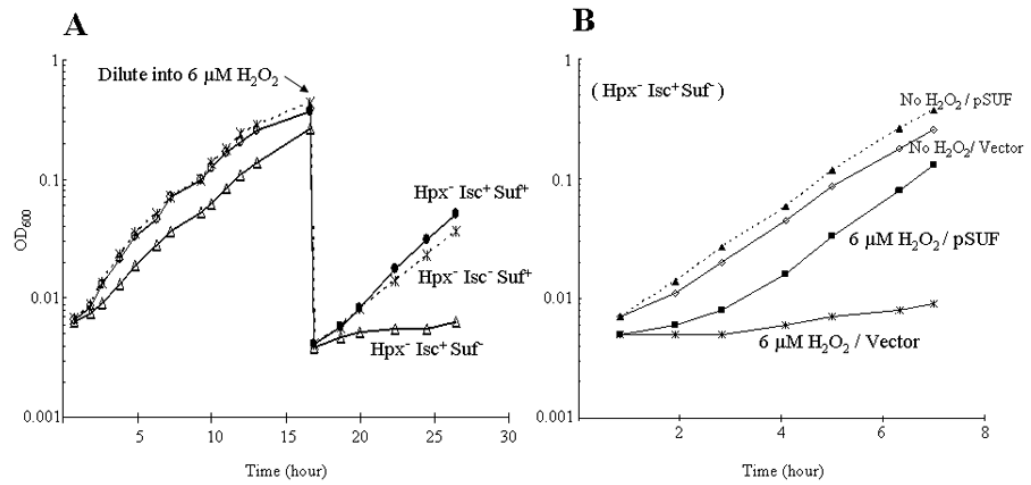


Figure 1. The Suf system is important for growth during micromolar H_2O_2 stress

(A) Cells were cultured in aerobic glucose medium. At the arrow, cells were diluted into fresh medium containing 6 μM H_2O_2 . (B) Anaerobic Hpx⁻ Isc⁺ Suf⁻ cells containing either pSUF (overexpressing the *suf* operon) or pWKS30 (an empty vector) were diluted into aerobic lactose minimal medium, with or without 6 μM H_2O_2 .

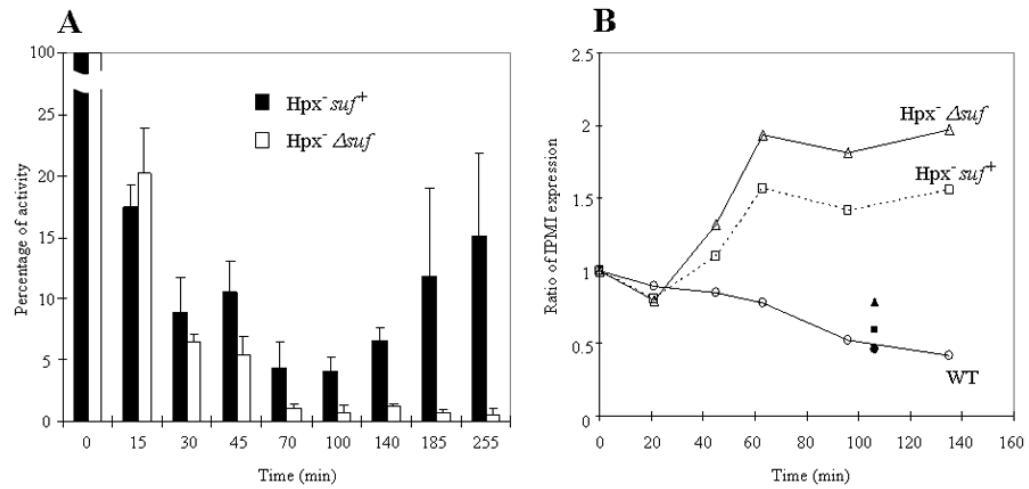


Figure 2. Induction of the Suf system is necessary to ensure IPMI activity during H₂O₂ stress (A) Anaerobic cultures were aerated at time zero, and cellular IPMI activity was measured during subsequent aerobic growth. 100% activity was approximately 0.6 U/mg. (B) The expression of *leuCD* was monitored by transcriptional *lacZ* fusions. Expression was normalized to anaerobic levels (time zero). Expression was shut off when aerobic medium contained leucine (solid symbols).

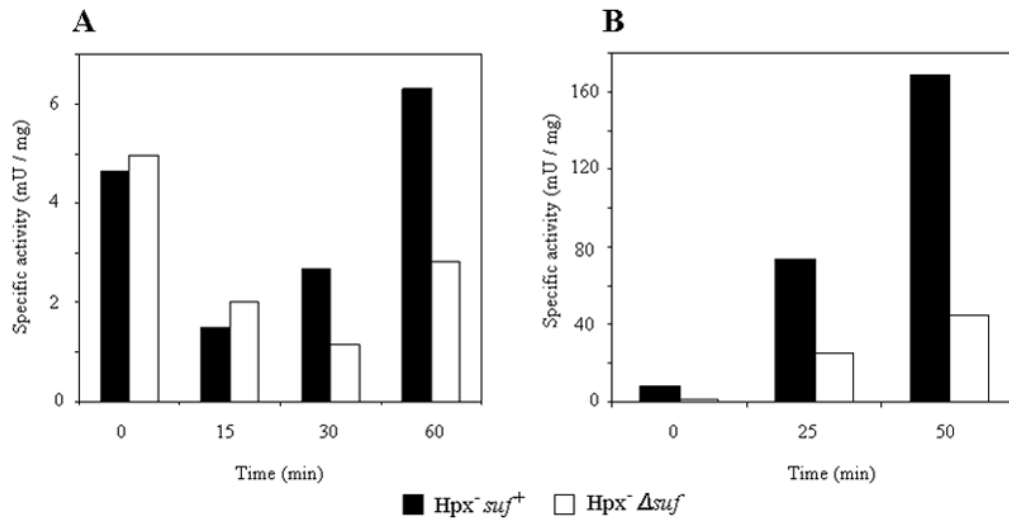


Figure 3. Suf was also necessary to support the activities of other labile [4Fe-4S] dehydratases Anaerobic log-phase cells (time zero) were diluted into aerobic media, and enzyme activity was monitored during subsequent aerobic growth. (A) Fumarase A activity in aerobic glucose/casamino acids medium supplemented with 20 μM H_2O_2 . (B) 6-phosphogluconate dehydratase activity. Anaerobic glucose-grown cells were washed and inoculated into aerobic gluconate medium at time zero, triggering the induction of 6-phosphogluconate dehydratase. The presence of casamino acids in both experiments ensured that cells continued to grow during the period of H_2O_2 stress. The data represent three independent trials.

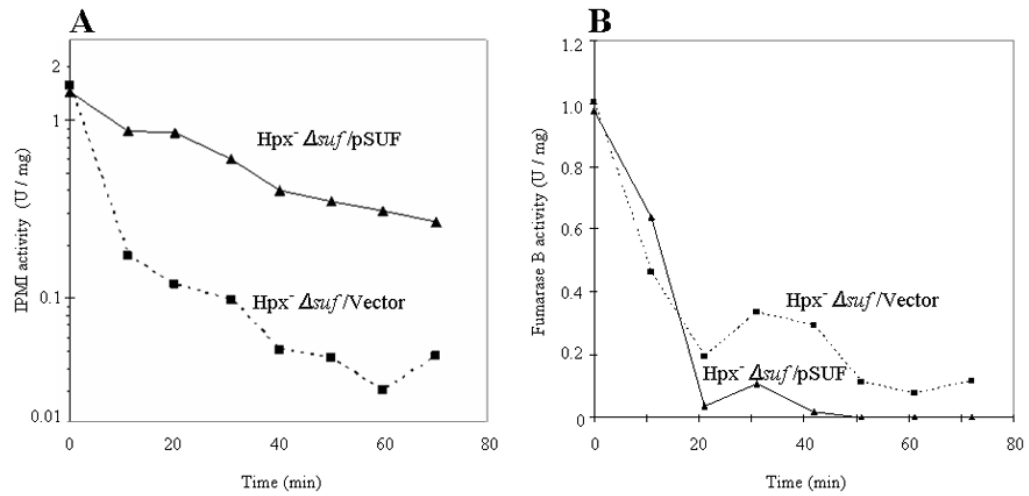


Figure 4. The Suf system repairs damaged IPMI but not fumarase B

Hpx⁻ Δsuf cells containing either pSUF or an empty vector were cultured anaerobically in lactose/18 amino acid medium that lacked leucine and cysteine. Chloramphenicol was added to block protein synthesis, and at time zero the cultures were aerated. IPMI (A) and fumarase B (B) activities were monitored. In the fumarase B experiment, 20 μM H_2O_2 was supplemented to the aerobic medium.

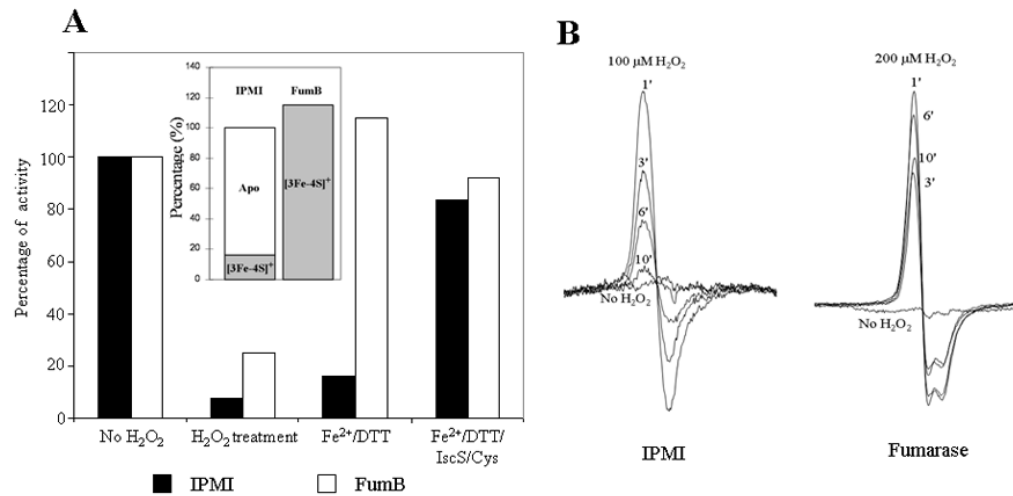


Figure 5. The oxidized Fe-S cluster of fumarase remains in a [3Fe-4S] form in vivo, while that in IPMI degrades further

(A) *Hpx⁻Δsuf* cells were grown anaerobically to allow synthesis of active IPMI and fumarase B. Chloramphenicol was added to block further synthesis, and cultures were then aerated to create H₂O₂ stress. For fumarase B experiments, 20 μM H₂O₂ was supplemented to the aerobic medium. After 20 min, lysates were prepared and assayed without further treatment, after incubation with ferrous iron/dithiothreitol, or after incubation with iron, DTT, and IscS/cysteine. Inset: Inferred status of the enzyme clusters upon harvesting. Initial activities were 1.6 U/mg (IPMI) and 1 U/mg (fumarase B). (B) *Hpx⁻Δsuf* cells that overproduce IPMI (left) or fumarase A (right) were harvested anaerobically, concentrated, and then exposed to 100 μM (IPMI) or 200 μM (fumarase) H₂O₂. The EPR signals represents the content of [3Fe-4S]⁺ clusters at subsequent time points. The signal is negligible in non-overproducing strains (not shown).

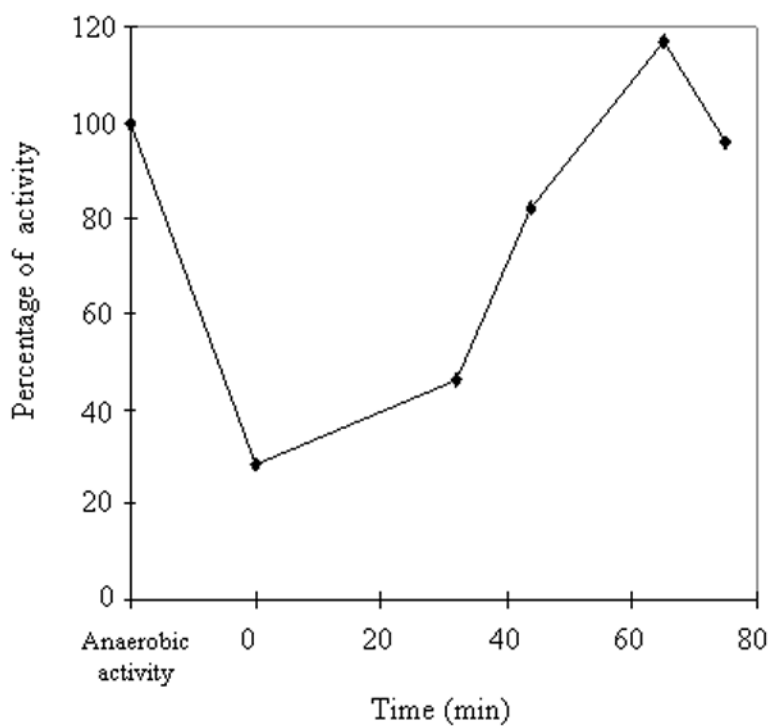


Figure 6. The repair of the $[3\text{Fe-4S}]^+$ cluster of fumarase A does not depend upon Suf
Chloramphenicol was added to anaerobic cultures of $\text{Hpx}^- \Delta\text{suf}$ cells, and they were aerated in medium containing $20 \mu\text{M H}_2\text{O}_2$ for 5 minutes. Catalase was then added, and the recovery of fumarase activity in the absence of H_2O_2 was monitored.

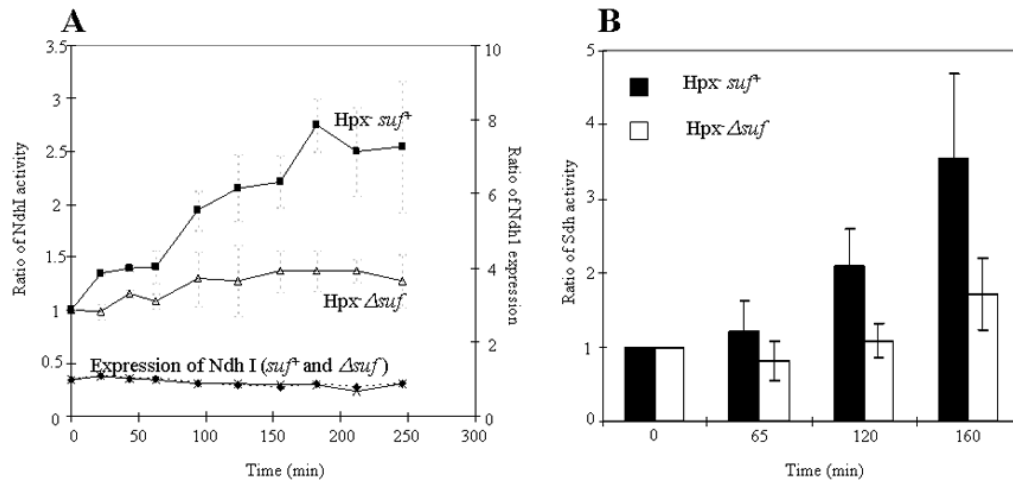


Figure 7. The Suf system is needed for the de novo assembly of iron-sulfur clusters during H₂O₂ stress

NADH dehydrogenase I (A) and succinate dehydrogenase (B) are induced when anaerobic cultures are aerated. Activities were measured at intervals after the aeration of Hpx⁻ *suf*⁺ and Hpx⁻ Δ *suf* cultures. Initial activities at time zero were approximately 30 mU/mg (NADH dehydrogenase I) and 40 mU/mg (succinate dehydrogenase). Panel A also indicates that *nuo* expression was independent of *suf* genotype.

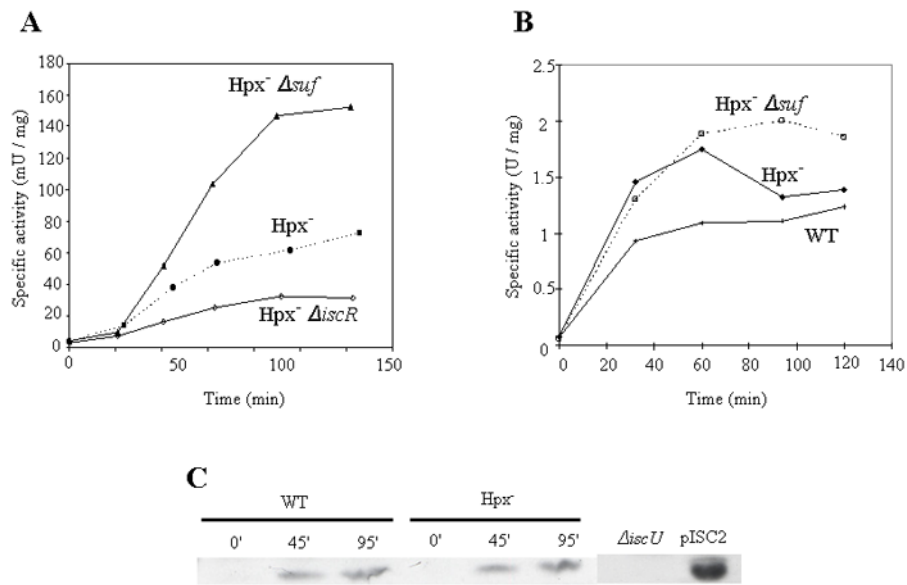


Figure 8. The failure of the Isc system during H₂O₂ stress is not due to a lack of Isc synthesis
 (A) Expression after aeration of a transcriptional *lacZ* fusion placed behind the *suf^{N1}* promoter that lacks the OxyR binding site. Induction by apo-IscR is greatest in a *suf* mutant.
 (B) Expression after aeration of a transcriptional *lacZ* fusion placed behind the *iscRSUA* promoter. (C) IscU polypeptide was detected by western blot analysis using an anti-IscU antibody during aerobic growth. The experiments were performed using glucose/casamino acids medium.

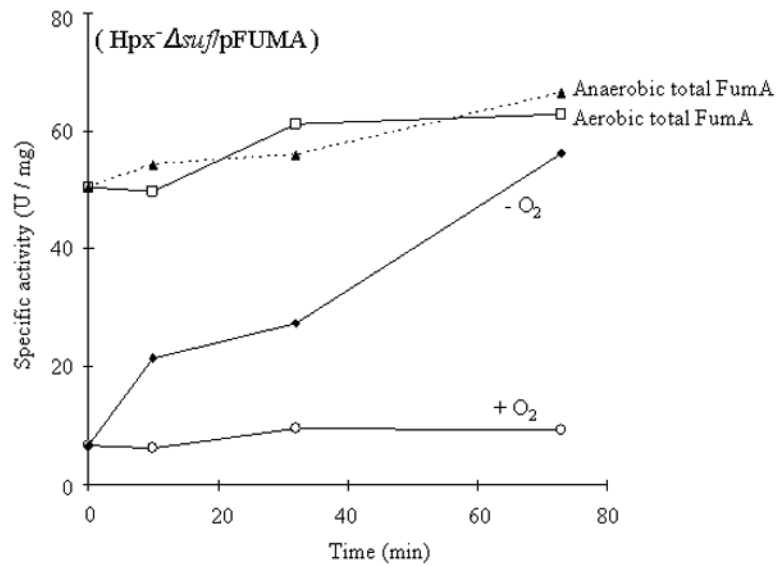


Figure 9. The inactivation of the Isc system by H₂O₂ is reversible

Fumarase A was strongly overexpressed in aerobic Hpx⁻Δsuf cells, forcing the accumulation of a large fraction of apoenzyme. Chloramphenicol was added to block protein synthesis. Cultures were then split and incubated either aerobically (+ O₂) or anaerobically with catalase (-O₂), and at intervals aliquots were harvested and assayed for fumarase activity. After cessation of H₂O₂ stress, Isc regained function and activated fumarase. Dashed lines: Total fumarase content was determined by treating the harvested extracts with ferrous iron, DTT, and IscS/cysteine.

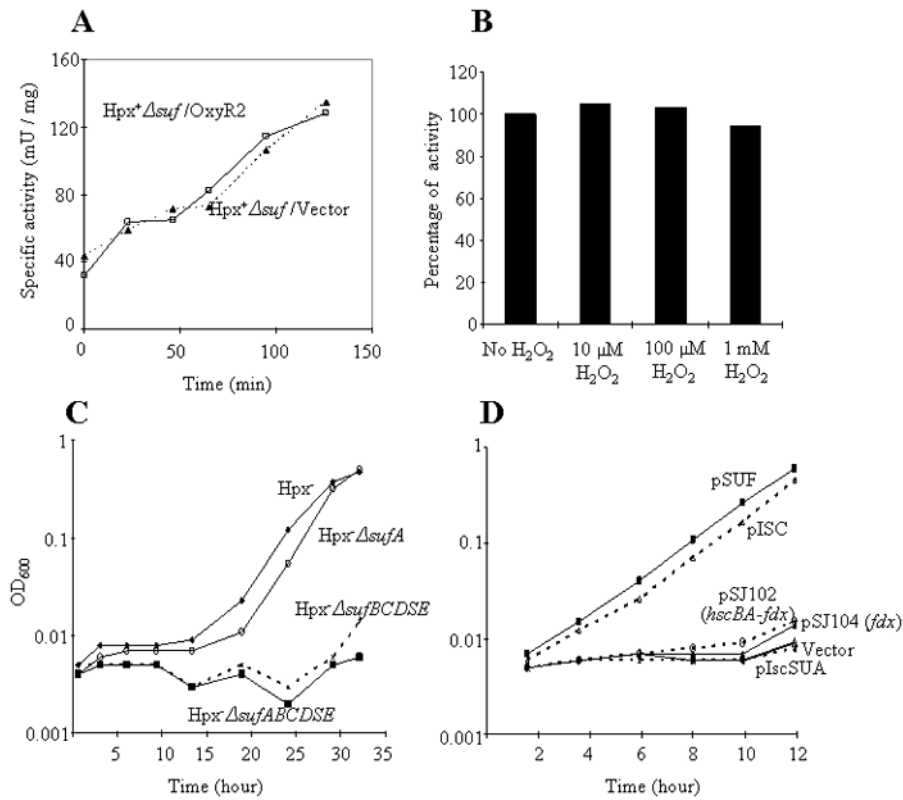


Figure 10. The inactivation of the Isc system is not due to OxyR induction or to the inactivation of IscS, IscA, or ferredoxin

(A) *Hpx⁺* cells (without H₂O₂ stress) were aerated at time zero. NADH dehydrogenase I activity was monitored in strains carrying a plasmid that strongly activates the OxyR regulon (pOxyR2) or an empty vector. (B) Purified IscS-(His)₆ was treated with the indicated concentrations of H₂O₂ for 5 min. Catalase was then added, and activity was assayed. (C) Growing cultures in anaerobic glucose medium were diluted at time zero into aerobic medium containing 8 μM H₂O₂, and growth was monitored. The *sufA* gene was not essential for outgrowth, due to the persistent function of IscA, but other *suf* genes were required. (D) The full Isc system must be overproduced to compensate for damage by H₂O₂. Growing cultures in anaerobic medium were induced by IPTG in order to express the entire *suf* operon (pSUF), the full *iscSUA hscAB-fdx* gene set (pISC), or the *hscBA-fdx, fdx*, or *iscSUA* components of the Isc system. All genes were controlled by the *lac* promoter, and robust expression was confirmed by visualization of the EPR signals of ferredoxin and/or IscA (text).

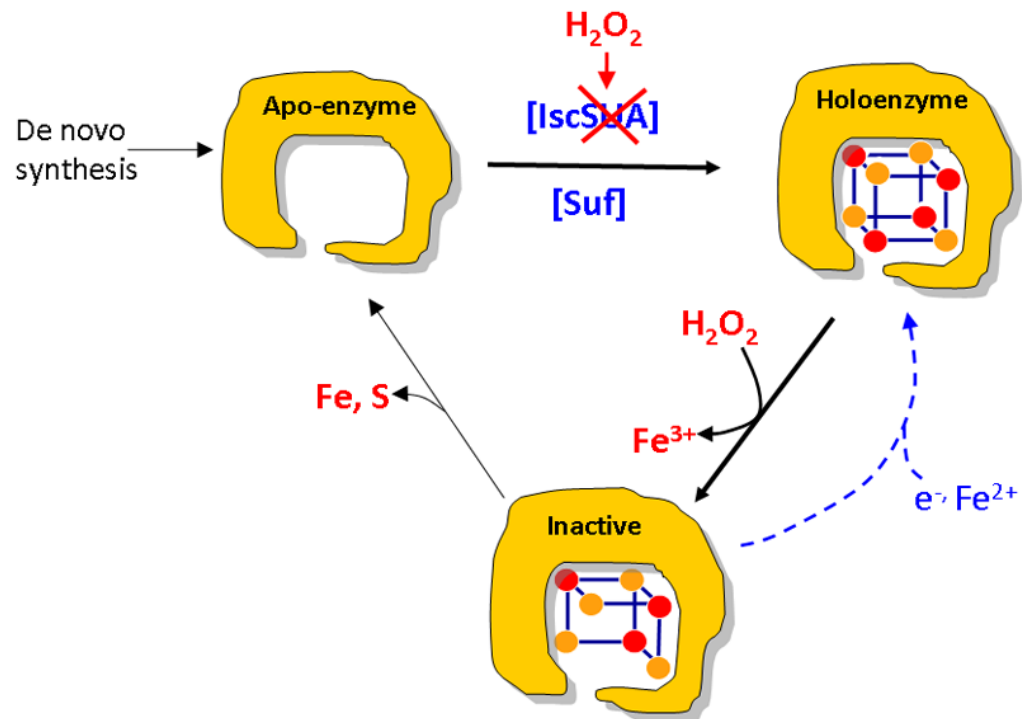


Figure 11. Iron-sulfur-cluster metabolism during H₂O₂ stress

Iron-sulfur-cluster biogenesis relies on the Isc system under routine conditions, and the Suf system is not expressed. However, H₂O₂ both poisons the Isc system and damages dehydratase clusters. In some enzymes the [3Fe-4S] product is stable and is repaired by simple reduction/metallation, while in other enzymes the clusters are further degraded. Strong induction of the Suf system by OxyR compensates for Isc inactivity, restoring de novo assembly and repairing oxidized apoenzymes.

Table 1

Strains	Genotype and characteristics	Source or ref.
MG1655	F ⁻ wild type <i>E. coli</i>	<i>E. coli</i> Genetic Stock Center
BW25113	<i>lacI rrnB ΔlacZ hsdK ΔaraBAD ΔrhaBAD</i>	(Datsenko & Wanner, 2000)
OD114	MG1655 <i>ΔhscA2::cat zff-208::Tn10</i>	(Djaman et al., 2004)
OD510	<i>Δ(sufABCDSE)19::kan~zdi57::Tn10 sodA25::MudPR13 sodB1- Δ2::kan</i>	(Djaman et al., 2004)
SJ89	MG1655 <i>Δ(sufABCDSE)19::kan~zdi57::Tn10</i>	P1(OD510) × MG1655
LC106	<i>ΔahpF::kan Δ(katG17::Tn10)1 Δ(katE12::Tn10)1</i>	(Seaver & Imlay, 2001a)
SJ15	LC106 <i>Δ(sufABCDSE)19::kan~zdi57::Tn10</i>	P1(SJ89) × LC106
SJ98	MG1655 <i>ΔlacZ1::cat</i>	This work
SJ100	LC106 <i>ΔlacZ1::cat</i>	P1(SJ98) × LC106
SJ102	SJ15 <i>ΔlacZ1::cat</i>	P1(SJ98) × SJ15
SJ130	MG1655 <i>Δ(lacZ1::cat)1</i>	This work
SJ108	LC106 <i>Δ(lacZ1::cat)1</i>	This work
SJ160	SJ15 <i>Δ(lacZ1::cat)1</i>	This work
SJ172	SJ130 <i>attλ::[pSJ501::sufA'-lac⁺]-cat</i>	This work
SJ186	SJ108 <i>attλ::[pSJ501::sufA'-lac⁺]-cat</i>	P1(SJ172) × SJ108
SJ57	LC106 <i>ΔhscA2::cat</i>	P1(OD114) × LC106
SJ283	BW25113 <i>Δ(OxyR binding site in PsufA)1::cat</i>	This work
SJ288	LC106 <i>Δ(OxyR binding site in PsufA)1::cat</i>	P1(SJ283) × LC106
SJ440	MG1655 <i>Δ(lacZ1::cat)1 attλ::[pSJ501::leuL'-lac⁺]-cat</i>	This work
SJ442	LC106 <i>Δ(lacZ1::cat)1 attλ::[pSJ501::leuL'-lac⁺]-cat</i>	P1(SJ440) × SJ108
SJ445	SJ15 <i>Δ(lacZ1::cat)1 attλ::[pSJ501::leuL'-lac⁺]-cat</i>	P1(SJ440) × SJ160
SJ40	BW25113 <i>Δ(fumC::cat)1 ΔfumB1::cat</i>	This work
SJ53	LC106 <i>Δ(fumC::cat)1 Δ(fumB1::cat)1</i>	This work
SJ65	SJ53 <i>Δ(sufABCDSE)19::kan~zdi57::Tn10</i>	P1(SJ89) × SJ53
SJ54	LC106 <i>Δ(fumCA::cat)1 Δ(fumB1::cat)1</i>	This work
SJ88	SJ54 <i>Δ(sufABCDSE)19::kan~zdi57::Tn10</i>	P1(SJ89) × SJ54
SJ90	BW25113 <i>ΔytfE1::cat</i>	This work
J1366	<i>ΔkatG17::Tn10 Δ(katE12::Tn10)1</i>	(Seaver & Imlay, 2001a)
SJ233	J1366 <i>ΔytfE1::cat</i>	P1(SJ90) × J1366
SJ91	SJ54 <i>ΔytfE1::cat</i>	P1(SJ90) × SJ54
SJ93	SJ88 <i>ΔytfE1::cat</i>	P1(SJ90) × SJ88
OD102	<i>hscA114::cat Φ(nuoAB-lacZ)</i>	(Djaman et al., 2004)
SJ109	SJ100 <i>Φ(nuoAB-lacZ)</i>	λ (OD102) × SJ100
SJ110	SJ102 <i>Φ(nuoAB-lacZ)</i>	λ (OD102) × SJ102
λJS2	<i>Φ(sdhC-lacZ) lacY⁺ lacA⁺</i>	(Shen & Gunsalus, 1997)
SJ150	MG1655 <i>Δfrd1::cat</i>	This work
SJ161	SJ108 <i>Δfrd1::cat</i>	SJ108 P1(SJ150)

Strains	Genotype and characteristics	Source or ref.
SJ163	SJ160 Δ frd1::cat	P1(SJ150) \times SJ160
SJ179	SJ161 Φ (sdhC-lacZ)	λ (λ JS2)x SJ161
SJ220	SJ163 Φ (sdhC-lacZ)	λ (λ JS2)x SJ163
SJ263	SJ130 att λ ::[pSJ501::sufA ^{NI-OxyR} -lac ⁺]-cat	This work
SJ276	SJ108 att λ ::[pSJ501::sufA ^{NI-OxyR} -lac ⁺]-cat	P1(SJ263) \times SJ108
SJ334	SJ160 att λ ::[pSJ501::sufA ^{NI-OxyR} -lac ⁺]-cat	P1(SJ263) \times SJ160
SJ272	BW25113 Δ iscR1::cat	This work
SJ291	SJ130 att λ ::[pSJ501::sufA ^{NI-OxyR} -lac ⁺] Δ iscR1::cat	P1(SJ272) \times SJ263
SJ293	SJ108 att λ ::[pSJ501::sufA ^{NI-OxyR} -lac ⁺] Δ iscR1::cat	P1(SJ272) \times SJ276
SJ332	SJ263 Δ (sufABCDSE)19::kan~zdi57::Tn10	P1(SJ89) \times SJ263
KI232	Δ (sodB::kan)1 Δ sodA-1	Laboratory stock
SJ259	KI232 Δ (lacZ1::cat)1	P1(SJ98) \times SJ297
SJ297	SJ259 Δ (sufABCDSE)19::kan~zdi57::Tn10	P1(SJ89) \times SJ259
SJ278	SJ259 att λ ::[pSJ501::sufA ^{NI-OxyR} -lac ⁺]-cat	P1(SJ263) \times SJ59
SJ295	SJ259 att λ ::[pSJ501::sufA ^{NI-OxyR} -lac ⁺] Δ iscR1::cat	P1(SJ272) \times SJ278
SJ336	SJ297 att λ ::[pSJ501::sufA ^{NI-OxyR} -lac ⁺]-cat	P1(SJ263) \times SJ297
SJ1010	SJ130 att λ ::[pSJ501::iscRS ⁺ -lac ⁺]-cat	This work
SJ1019	SJ108 att λ ::[pSJ501::iscRS ⁺ -lac ⁺]-cat	P1(SJ1010) \times SJ108
SJ1021	SJ160 att λ ::[pSJ501::iscRS ⁺ -lac ⁺]-cat	P1(SJ1010) \times SJ160
JI370	Δ ahpF::kan	(Seaver & Imlay, 2001a)
SP65	Δ mhpC281::Tn10 lacY1 Δ dps-1::cat	(Park et al., 2005)
SJ181	JI370 Δ dps-1::cat	P1(SP65) \times JI370
SJ183	SJ181 Δ (sufABCDSE)19::kan~zdi57::Tn10	P1(SJ89) \times SJ181
SJ1001	BW25113 Δ sufA1::cat	This work
SJ1004	BW25113 Δ (sufBCDSE)1::cat	This work
SJ1023	BW25113 Δ (sufSE)1::cat	This work
OD112	Δ iscA112::cat	(Datsenko & Wanner, 2000)
SJ1013	LC106 Δ iscA112::cat	P1(OD112) \times LC106
SJ1015	LC106 Δ sufA1::cat	P1(SJ1001) \times LC106
SJ1017	LC106 Δ (sufBCDSE)1::cat	P1(SJ1004) \times LC106
SJ1042	LC106 Δ (sufSE)1::cat	P1(SJ1023) \times LC106
SJ1046	LC106 Δ (sufA1::cat)1 Δ iscA112::cat	P1(OD112) \times SJ1015
SJ424	BW25113 Δ bfr-1::cat	This work
SJ448	BW25113 Δ (bfd bfr)-1::cat	This work
KCI540	LC106 Δ cyaY1::cat	Laboratory stock
JRG2953	W3110 Δ bfr::kan Δ fm::spec	J.R. Guest
SJ428	LC106 Δ bfr-1::cat	P1(SJ424) \times LC106
SJ432	SJ15 Δ bfr-1::cat	P1(SJ424) \times SJ15
SJ459	LC106 Δ (bfd bfr)-1::cat	P1(SJ448) \times LC106

Strains	Genotype and characteristics	Source or ref.
SJ453	LC106 <i>Afm::spec</i>	P1(JRG2953) × LC106
SJ463	SJ15 Δ <i>cyaY1::cat</i>	P1(KCI540) × SJ15
SJ461	SJ453 Δ (<i>bfd bfr</i>)-1:: <i>cat</i>	P1(SJ448) × SJ453
SJ466	SJ461 Δ (<i>sufABCDSE</i>)19:: <i>kan~zdi57::Tn10</i>	P1(SJ89) × SJ461
SJ473	SJ453 Δ (<i>bfd bfr</i>)-1:: <i>cat</i>)1 Δ <i>cyaY1::cat</i>	P1(KCI540) × SJ461
SJ487	SJ473 Δ (<i>sufABCDSE</i>)19:: <i>kan~zdi57::Tn10</i>	P1(SJ89) × SJ473
Plasmid		
pCP20	FLP expression plasmid; Amp ^r , temperature-sensitive replication and FLP synthesis	(Cherepanov & Wackernagel, 1995)
pKD3	Template plasmid; amp, FRT-flanked <i>cat</i>	(Datsenko & Wanner, 2000)
pKD46	λ Red recombinase (γ , β , and <i>exo</i>) expression plasmid; amp, ara- inducible expression, temperature-sensitive replication	(Datsenko & Wanner, 2000)
pCKR101	P _{tac} - <i>lacI</i> ^q P _{tac} polylinker Amp ^r (20–50 copies per cell)	Jeff Gardner
pWKS30	P _{tac} polylinker Amp ^r (6–8 copies per cell)	(Wang & Kushner, 1991)
pSUF1	pWKS30 containing <i>sufABCDSE</i>	This work
pISC1	pWKS30 containing <i>iscSUA hsc BhscA fdx iscX</i>	This work
pISC2	pCKR101 containing <i>iscSUA hscB hscA fdx iscX</i>	This work
pIscSUA	pWKS30 containing <i>iscSUA</i>	This work
pSJ102	pWKS30 containing <i>hscB hscA fdx iscX</i>	This work
pSJ104	pWKS30 containing <i>fdx iscX</i>	This work
pET15b	P _{T7} polylinker Amp ^r	Lab stock
pIscS-(His) ₆	pET15b containing <i>iscS</i>	This work
pTrcfdx	pTrc99 (P _{trc} polylinker Amp ^r) containing <i>fdx</i>	(Ta & Vickery, 1992)
pFUMA	pCKR101 containing <i>fumA</i>	(Jang & Imlay, 2007)
pAH125	CRIM reporter plasmid (Km ^r)	(Haldimann & Wanner, 2001)
pSJ501	pAH125 derivative, in which the Km resistant marker has been replaced with a <i>cat</i> gene.	This work
pAH57	CRIM helper plasmid	(Haldimann & Wanner, 2001)
pACYC184	Tet ^r Cm ^r	(Kullik et al., 1995)
pGS058	pACYC184 containing <i>oxyR2</i> [A233V]	(Kullik et al., 1995)

Table 2

Plasmid	Primer sequences
pSUF1	Forward 5'-ATATCGAATTCTAAGTAAGAGGTAAATCGATGGACA-3' Reverse 5'-CATGGATCTAGATTAGCTAAGTGCAGCGGC-3'
pISC1 pISC2	Forward 5'-ATATCGAATTCTTTAATACGGAGTTTATAGAGCA-3' Reverse 5'-CATGGATCTAGA TTATTCGGCCTCGTCCAG-3'
pIscS-(His) ₆	Forward 5'-ATCGATCCATATGAAATTACCGATTTATCTCGAC-3' Reverse 5'-CATATAGGATCCTTAATGATGAGCCCCATTCGATG-3'
pIscSUA	Forward 5'-ATATCGAATTCTTTAATACGGAGTTTATAGAGCA-3' Reverse 5'-CATGGATCTAGATCAAACGTGGAAGCTTTC-3'
pSJ104	Forward 5'-ATATCGAATTCTTTAACGCAGCCCTGAGAATGTT-3' Reverse 5'-CATGGATCTAGA TTATTCGGCCTCGTCCAG-3'
pSJ107	Forward 5'-ATATCGAATTCTTTAACCGTGGACGAGGTTTAAT-3' Reverse 5'-CATGGATCTAGA TTATTCGGCCTCGTCCAG-3'
pPsuf	Forward 5'-ATATGCCTGCAGCTTAAGGGTTTTCTATTTC-3' Reverse 5'-TATACCGGTACCCATCGATTACCTCACTTC-3'
pPsufNI	Forward 5'-ATATGCCTGCAGCTAACAATGAGATACCTAATTC-3' Reverse 5'-TATACCGGTACCCATCGATTACCTCACTTC-3'
pPiscR	Forward 5'-ATATGCCTGCAGAGGTCGGATAAAGGCGTTC-3' Reverse 5'-TATACCGGTACCCATGTCTTACTTCACCTC-3'
pPiscRS	Forward 5'-ATATGCCTGCAGAGGTCGGATAAAGGCGTTC-3' Reverse 5'-TATACCGGATCCCATGTCTATAAACTCC-3'
pPleuL2	Forward 5'-ATATGCCTGCAGTACTTAACTCCAAGTCA-3' Reverse 5'-TATACCGGTACCCTGTTACCGTCGCGCAATG-3'