Micromolar Intracellular Hydrogen Peroxide Disrupts Metabolism by Damaging Iron-Sulfur Enzymes*

Soojin Jang and James A. Imlay

From the Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

An Escherichia coli strain that cannot scavenge hydrogen peroxide has been used to identify the cell processes that are most sensitive to this oxidant. Low micromolar concentrations of H$_2$O$_2$ completely blocked the biosynthesis of leucine. The defect was tracked to the inactivation of isopropylmalate isomerase. This enzyme belongs to a family of [4Fe-4S] dehydratases that are notoriously sensitive to univalent oxidation, and experiments confirmed that other members were also inactivated. In vitro and in vivo analyses showed that H$_2$O$_2$ directly oxidized their solvent-exposed clusters in a Fenton-like reaction. The oxidized cluster then degraded to a catalytically inactive [3Fe-4S] form. Experiments indicated that H$_2$O$_2$ accepted two consecutive electrons during the oxidation event. As a consequence, hydroxyl radicals were not released; the polypeptide was undamaged; and the enzyme was competent for reactivation by repair processes. Strikingly, in scavenger-deficient mutants, the enzyme was competent for reactivation by repair processes. Strikingly, in scavenger-deficient mutants, the enzyme was competent for reactivation by repair processes.

This result demonstrates that aerobic organisms must synthesize H$_2$O$_2$ scavengers to avoid poisoning their own pathways. The extreme vulnerability of these enzymes may explain why many organisms, including mammals, deploy H$_2$O$_2$ to suppress microbial growth.

Virtually all organisms express peroxidases and catalases to protect themselves from hydrogen peroxide. H$_2$O$_2$ is continuously formed by the autoxidation of redox enzymes (reviewed in Ref. 1), and scavenging enzymes have originally evolved to protect cells against these internal sources of H$_2$O$_2$. The peroxidases and catalases are sufficiently abundant and active that they probably drive the steady-state level of intracellular H$_2$O$_2$ into the low nanomolar range (2). Nevertheless, it is widely suspected that even this dose of H$_2$O$_2$ may compose a chronic, low level stress that gradually debilitates cells and, in higher organisms, drives the deterioration of tissue function as part of the aging process.

Exogenous H$_2$O$_2$ rapidly diffuses across cell membranes (2) and can impose a much more acute stress on cells; accordingly, it is often used as a biological weapon. For example, H$_2$O$_2$ is formed by phagocytes and may accumulate to $10^{-4}$ M inside phagosomes that have engulfed invading bacteria. Lactic acid bacteria suppress the growth of competitors by releasing H$_2$O$_2$ as a primary metabolic product, achieving millimolar concentrations in laboratory cultures. And redox-cycling antibiotics, which are produced as microbicides by both plants and bacteria, suffice target organisms with a continuous stream of H$_2$O$_2$.

If we wish to understand the severity and nature of the stress that H$_2$O$_2$ imposes upon cells, we must identify the biomolecules with which it primarily reacts. This problem has not been easy to solve. In vitro studies have shown that H$_2$O$_2$ can oxidize methionine (3) and cysteine (4) residues, but the rates at which it does so suggest that these types of damage will be scant at physiological doses of H$_2$O$_2$, unless the surrounding polypeptide context somehow strongly activates the residues. Reactions between H$_2$O$_2$ and loosely bound iron generate hydroxyl radicals (Reaction 1) and are suspected of being involved in protein carbonylation, lipid peroxidation, and DNA oxidation (5).

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Fe^{2+} + H_2O_2 \rightarrow HO^+ + OH^- + Fe^{3+}
\]

REACTION 1

Early measurements indicated that this reaction (the Fenton reaction) is relatively slow as well (6), prompting some workers to question its significance in real-world scenarios (discussed in Ref. 5); however, subsequent work revealed that anionic ligands activate ferrous iron to the point that it reacts quickly with micromolar H$_2$O$_2$ (7, 8).

An alternative approach to pinpointing the important targets of H$_2$O$_2$ is to expose cells to increasing doses in a way that identifies the first cell processes to fail. Escherichia coli and other organisms have calibrated their defensive systems to detect submicromolar levels of H$_2$O$_2$ (2, 9), so we anticipate that these low concentrations are sufficient to threaten the most sensitive biomolecules. Unfortunately, if scavenging enzymes are active, it is difficult to impose such a low dose of H$_2$O$_2$ over an extended period of time because the enzymes will degrade the H$_2$O$_2$ and end the stress. Therefore, these experiments are most easily conducted with scavenger-deficient mutants. We have constructed E. coli strains that lack peroxidase and catalase activities (10). These mutants grow at wild-type rates in anaerobic environments, but when they are exposed to oxygen, they grow at reduced rates in complex medium and fail to grow at all in minimal medium. The former defect is due, at least in part, to Fenton-mediated DNA damage (8). The second defect stems from problems with biosynthetic pathways. In this study, we identified the mechanism by which micromolar H$_2$O$_2$ blocks...
Damage to [4Fe-4S] Clusters by \( \text{H}_2\text{O}_2 \)

**TABLE 1**

| Strains and plasmids used in this study | Genotype/characteristics | Source/Ref. |
|----------------------------------------|--------------------------|-------------|
| **Strain** | **Wild-type E. coli** | | |
| MG1655 | \( \Delta(\text{katG}17:\text{Tn}10)\) | 45 |
| LC106 | \( \Delta(\text{ahpC}::\text{ahpF})\) | 10 |
| JH400 | \( \Delta(\text{src}::\text{araC})\) | |
| OD570 | \( \Delta(\text{fumCZ}::\text{cm})\) | 19 |
| OD571 | \( \Delta(\text{fumCA}::\text{cm})\) | 19 |
| SI19 | \( \Delta(\text{fumCZ}::\text{cm})\) in LC106 | This study |
| SI20 | \( \Delta(\text{fumAC}::\text{cm})\) in LC106 | This study |
| SI34 | \( \Delta(\text{fumCZ}::\text{cm})\) in BW2113 | This study |
| SI37 | As LC106 plus \( \Delta(\text{fumC}::\text{cm})\) | This study |
| SI38 | As LC106 plus \( \Delta(\text{fumAC}::\text{cm})\) | This study |
| BW25113 | lac\(\text{P}_{\text{r}}\) rnbT74, \(\text{lacZ}_{\text{B}}\) hsdR514, \(\Delta\text{araBAD}_{\text{A}1231}\) \(\Delta\text{hrBAD}_{\text{A}1278}\) | 12 |
| **Plasmid** | \(\text{pWKS30}\) | 18 |
| | \(\text{pLEUCD2}\) | This study |
| | \(\text{pCR101}\) | This study |
| | \(\text{pLEUCD3}\) | This study |
| | \(\text{pFUMA}\) | This study |
| | \(\text{pCP20}\) | 46 |
| | \(\text{pKD3}\) | 12 |
| | \(\text{pKD46}\) | 12 |

leucine biosynthesis, and we found that this class of injury affects multiple pathways in the cell.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Conditions**—The strains and plasmids used in this study are listed in Table 1. Anaerobic cultures were grown in an anaerobic chamber (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 (11), 0.2% glucose, 1 mm MgCl\(_2\), 5 mg/liter thiamine, 0.5 mm histidine, 0.5 mm phenylalanine, 0.5 mm tyrosine, and 0.5 mm tryptophan. Histidine was always added to the media because the parent strain, MG1655, is a histidine auxotroph (11). To create a null mutation of fumA, overnight cultures of hydroperoxidase-deficient (Hpx\(^-\)) were diluted to OD\(_{570}\) = 0.005 in fresh anaerobic glucose minimal medium. Cells were then grown anaerobically to OD\(_{571}\) = 0.930.

**Aerobic Cell Growth**—To ensure that cells were growing anaerobically prior to dilution into aerobic medium.

**Enzyme Assays**—Cell extracts were prepared by suspending and sonicating cells in anaerobic buffers inside an anaerobic chamber. Isopropylmalate isomerase (IPMI) activity was measured by monitoring the decrease in the absorbance (235 nm) of citraconate (Sigma) (13), an analog of isopropylmalate; reactions contained 100 mM Tris-Cl (pH 7.6) and 0.4 mM citraconate. Fumarase activity was determined from the appearance of fumarate (250 nm) in a reaction containing 50 mM sodium phosphate (pH 7.4) and 50 mM malate (Sigma) (14). To assay 6-phosphogluconate dehydratase, lysates were prepared from cultures grown in minimal medium containing 0.2% gluconate. Turnover of 6-phosphogluconate dehydratase produces pyruvate; its formation (in 50 mM Tris-Cl (pH 7.65)) was determined in a second reaction catalyzed by lactate dehydrogenase (Sigma) (15). To assay NADH dehydrogenase I, inverted membrane vesicles were isolated from extracts after sonication in 50 mM MES (pH 6.0) (16). NADH dehydrogenase I oxidizes deamino-NADH (340 nm) in 50 mM potassium phosphate buffer (pH 7.8), whereas NADH dehydrogenase II does not. Aconitase was assayed by the conversion of isocitrate to aconitate (17).

**Plasmid Construction**—The leuCD open reading frame was PCR-amplified from *E. coli* MG1655 using the forward primer 5’-ATATCGATCTTACTGATCGCTTCGCGTCAATTCGAC-3’ and the reverse primer 5’-GGGATCCGGCAGCAGGCGCTG-3’ and the reverse primer 5’-GGGATCCGGCAGCAGGCGCTG-3’. To construct the pLEUCD2 plasmid, the PCR products were digested with XbaI and EcoRI and cloned into the pWKS30 vector (18) behind the lac promoter. The plasmid was confirmed by restriction analysis and IPMI assay. Hpx\(^-\) cells containing pLEUCD2 or pWX30 were cultured in lactose minimal medium with histidine, aromatic amino acids, and 50 \(\mu\)g/ml ampicillin. This plasmid was used in complementation experiments; the leuCD genes were induced 3-fold above wild-type levels by 1 mm isopropyl \(\beta\)-d-thiogalactopyranoside, which was added to both anaerobic precultures and final aerobic cultures.

For EPR experiments and purification purposes, IPMI and fumarase A were strongly overproduced by expression from a tac promoter. The leuCD genes were excised from pLEUCD2 with XbaI and EcoRI and cloned into the pCKR101 vector to construct pLEUCD3. The fumA open

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2 The abbreviations used are: Hpx\(^-\), hydroperoxidase-deficient; IPMI, isopropylmalate isomerase; MES, 4-morpholineethanesulfonic acid.
reading frame was PCR-amplified from *E. coli* MG1655 using the forward primer 5’-ATATCGAATCTTTAACATA- AAACAAACGGCGTAAGT-3’ and the reverse primer 5’-CATGGATCTAGATTAATTTACCACAGCGGG- TGCAATTG-3’. PCR products were digested with XbaI and EcoRI and cloned into the pCRK101 vector to construct pFUMA. Cells containing the plasmids were cultured in glucose minimal medium with histidine, aromatic amino acids, and 50 μg/ml ampicillin. These plasmids overproduced IPMI and fumarase A >60-fold above wild-type levels.

**Inactivation of Enzymes**—Hpx− cells were grown anaerobically to A600 = 0.2. H2O2 was added to the cultures when they were aerated. At designated time points, aliquots were removed; catalase was added to 200 units/ml; and cells were returned to the anaerobic chamber for lysis and assay.

In *vivo* inactivation of IPMI by endogenous H2O2 was initiated by aerating heretofore anaerobic cultures without any addition of exogenous H2O2. Under these conditions, cells steadily generate H2O2. The H2O2 equilibrates so quickly across membranes that, in Hpx− cultures, the intracellular H2O2 concentration is essentially equivalent to the extracellular concentration (2). Extracellular H2O2 was measured directly (250 μM H2O2 is readily detectable using the forward primer 5’-AACCAAACCAGGCAGTAAGTG-3’ and the reverse primer 5’-CATGGATCTAGATTAATTTACCACAGCGGG- TGCAATTG-3’). PCR products were digested with XbaI and EcoRI and cloned into the pCRK101 vector to construct pFUMA. Cells containing the plasmids were cultured in glucose minimal medium with histidine, aromatic amino acids, and 50 μg/ml ampicillin. These plasmids overproduced IPMI and fumarase A >60-fold above wild-type levels.

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Inactivation of enzymes *in vitro* was accomplished by the addition of H2O2 to lysates or to purified enzyme in anaerobic buffer. The H2O2 was subsequently removed by catalase prior to anaerobic assay. In some cases, damaged iron-sulfur clusters were chemically rebuilt by incubation with 50 μM Fe(NH4)2(SO4)2 (Sigma) and 2.5 mM dithiothreitol (Sigma) (17) at room temperature. Fe(NH4)2(SO4)2 (Sigma) and 2.5 mM dithiothreitol (Sigma) (17) at room temperature.

**EPR Analysis**—*In vivo* EPR samples were prepared with Hpx− cells overproducing IPMI or fumarase A. To overexpress the structural genes, 1 mM isopropyl β-D-thiogalactopyranoside was added when the cells reached A600 = 0.2. After another 2 h of incubation, the cells were harvested by centrifugation, and the cell pellets were resuspended at 1/50th of the original culture volume in 10% glycerol. The resuspended cells were incubated with H2O2 for 1 min at 37 °C. The cell suspension (250 μl) was then transferred into an EPR tube and frozen in dry ice. EPR spectra of [3Fe-4S]+ clusters were obtained at the following settings: microwave power, 1 milliwatt; microwave frequency, 9.05 GHz; modulation amplitude, 12.5 G at 100 KHz; and time constant, 0.032. Iron concentrations were quantified using standard solutions of FeCl3 (Sigma) in 50 mM Tris-Cl containing 10 mM Mg2+ and 1 mM desferrioxamine. Desferrioxamine binds both ferric and ferrous iron and triggers the oxidation of the latter species. Thus, the EPR method did not distinguish whether the released iron was in the ferric or ferrous form.

Ferene is commonly used to detect ferrous iron released by metalloenzymes, but we found that ferene itself directly inactivated fumarase A. Dipyrindyl did not. Dipyrindyl binds ferrous iron in a complex that exhibits an absorbance maximum at 522 nm (21); we determined that this complex cannot be oxidized by H2O2. To detect Fe2+ released during cluster decomposition, 1 mM dipyrindyl (Sigma) was added to 18 μM fumarase A prior to H2O2 addition. Control experiments confirmed that this concentration of dipyrindyl captured ferrous iron before H2O2 could oxidize it: when Fe(NH4)2(SO4)2 was added to the H2O2-containing reaction mixture, we were able to quantitatively recover it as a dipyrindyl chelate, and we could accurately detect as little as 2 μM.

**Protein Mass Spectroscopy**—Fumarase A (3 μM) was treated with 5 μM H2O2 for 2 min at room temperature. The reaction was terminated by the addition of 0.1 unit/μl catalase. Assays showed that >98% of the enzyme had been inactivated. For mass spectroscopic analysis, 2 μg of sample was desalted using the Genotech Perfect-FOCUS two-dimensional sample cleanup kit. The desalted sample was suspended in 25 mM ammonium bicarbonate containing 12.5 μg/ml trypsin and incubated for 12 h at 37 °C. The sample was then dried, suspended in 5% acetonitrile and 0.1% formic acid, and injected into a Waters Q-Tof quadrupole time-of-flight mass spectrometer via a high pressure liquid chromatography interface. Pep-
Damage to [4Fe-4S] Clusters by H₂O₂

Hydroperoxidase mutants that cannot scavenge H₂O₂ (katG katE ahp; here denoted Hpx⁻) grow well in anaerobic glucose minimal medium but stop growing when they are aerated. The cells resume growth when aromatic amino acids are supplied, indicating that endogenously formed H₂O₂ poisons some step in the aromatic biosynthetic pathway. The mechanism is unknown and is the subject of a separate investigation. However, aromatic supplements do not fully restore these mutants to a wild-type growth rate, and the addition of small amounts of exogenous H₂O₂ exacerbates the residual defect. Eight micromolar H₂O₂ completely blocked growth in this medium (Fig. 1A). Supplementation with casamino acids restored growth, implying that stasis was a result of a second amino acid biosynthetic defect.

Hpx⁻ growth in the presence of H₂O₂ was improved by supplementation with exogenous α-ketoisocaproate, the final intermediate in the leucine pathway, but not by α-ketoisovalerate, the first one (data not shown). There are five reactions between the two intermediates catalyzed by four enzymes. We anticipated that H₂O₂ might inhibit or inactivate one of them. Indeed, the growth defect was partially relieved by a plasmid that overproduces IPMI 3-fold (Fig. 1B), suggesting that this is the rate-limiting enzyme in the pathway during H₂O₂ stress.

Enzyme assays confirmed that IPMI lost activity rapidly when 8 μM H₂O₂ was added to cultures (Fig. 2A). Furthermore, significant enzyme inactivation occurred upon aeration even without the addition of exogenous H₂O₂ (Fig. 2B), indicating that H₂O₂ was sufficient to poison a substantial fraction of IPMI. Thus, this enzyme is exquisitely sensitive, and scavenging enzymes are needed to protect it from endogenously formed H₂O₂.

The Nature of IPMI Damage—IPMI activity was lost when H₂O₂ was added to anaerobically prepared Hpx⁻ extracts (Fig. 3A), indicating that inactivation occurred by direct action of H₂O₂ upon the enzyme. Catalase protected completely (data not shown).

IPMI is a dehydratase, and its protein sequence suggests that it belongs to a family of enzymes that employ [4Fe-4S] clusters as active-site catalysts (22). The solvent-exposed clusters of these enzymes both coordinate substrate and act as Lewis acids in abstracting the hydroxide anion from the bound substrate (23). These enzymes are typified by aconitase, and they are notoriously sensitive to inactivation by univalent oxidants such as superoxide and ferricyanide (15, 24–27). These agents abstract a single electron from the cluster, converting it to a [4Fe-4S]³⁺ form. The cluster is unstable at that valence and releases the substrate-binding iron atom as Fe²⁺ so that the residual cluster is left in a [3Fe-4S]⁺ form that lacks the key iron atom and is catalytically inactive.

Consistent with this model, the addition of citraconate, a pseudosubstrate of IPMI, protected the enzyme from H₂O₂ in vitro, suggesting that H₂O₂ must directly contact the cluster to inactivate the enzyme (data not shown). Damaged clusters can often be reassembled chemically by treatment with dithiothreitol and ferrous iron; when damaged IPMI...
was subjected to this protocol, 60% of the activity was regained within 3 min (Fig. 3B).

IPMI was overexpressed in Hpx⁻ cells, and cells were then exposed to H₂O₂. A strong [3Fe-4S]⁺ signal appeared (Fig. 3C), which was absent in non-overproducing controls. This result confirmed both that IPMI has a cluster and that it is destroyed by H₂O₂. In fact, ~85% of the IPMI activity was recovered when the extracts from H₂O₂-exposed cells were treated with dithiothreitol and ferrous iron.

Other [4Fe-4S] Dehydratases Are Similarly Sensitive to H₂O₂—Other cluster-containing dehydratases (6-phosphogluconate dehydratase and fumarases A and B) also lost activity when they were exposed to low concentrations of H₂O₂ in vitro (Fig. 4, A–C). In each case, full activity could be restored by subsequent treatment with dithiothreitol and ferrous iron (data not shown). Furthermore, a strong [3Fe-4S]⁺ signal was detected when H₂O₂ was added to Hpx⁻ cells overproducing fumarase A (Fig. 5). Thus, it appears that H₂O₂ efficiently damages the clusters of all members of this enzyme family.

Glucose medium, which we employed for our initial growth experiments, does not demand that E. coli process substrate through its tricarboxylic acid cycle to generate ATP; therefore, growth would not have been affected by the inactivation of aconitase or fumarase. However, the Hpx⁻ strain exhibited a severe growth defect when it was cultured in malate medium (Fig. 6). This result indicates that the submicromolar H₂O₂ that is generated by endogenous processes is sufficient to debilitate this pathway.

Iron-sulfur clusters are also used by respiratory enzymes to transfer electrons between active sites. The NADH dehydrogenase I complex is characteristic of these enzymes, as it contains at least nine iron-sulfur clusters (28). However, even 5 mM H₂O₂ was unable to diminish its activity (data not shown). Because these clusters are buried within polypeptide, the implication is that H₂O₂ can oxidize only those clusters that it can contact directly.
The Mechanism of Cluster Inactivation—IPMI is a two-subunit enzyme that dissociates during purification. Therefore, fumarase A and aconitase A were chosen for purification and further examination. The isolated enzymes were acutely sensitive to H$_2$O$_2$, exhibiting inactivation rate constants of 4 x 10$^{-3}$ and 3 x 10$^{-2}$ M$^{-1}$ s$^{-1}$, respectively, at 0 °C. The fumarase inactivation constant was 0.5–1 x 10$^{-5}$ M$^{-1}$ s$^{-1}$ at 25 °C, and inactivation at 37 °C was too fast for us to measure.

Strikingly, the fumarase rate constants were orders of magnitude higher than the apparent constant that we calculated in vivo using Hpx$^{-}$ mutants. We suspected that substrates protect the enzyme inside the cell. Indeed, both malate and fumarate fully protected fumarase when they were added at saturating concentrations (Fig. 7). The doses needed for half-maximal protection (0.4 mM for malate and 0.3 mM for fumarate, measured at 0 °C) were in reasonable agreement with the $K_m$ values of the enzyme for those substrates (0.7 and 0.6 mM, respectively, at 37 °C) (29). Thus, it is likely that the inactivation of the enzyme in vivo is tempered by the consequent accumulation of substrate. These data also support the suspicion that H$_2$O$_2$ must intimately contact the cluster to inactivate it.

The standard model of univalent cluster oxidation posits that a [3Fe-4S]$^{2+}$ form is initially generated with release of one ferrous ion (27). However, if the oxidant is H$_2$O$_2$, then a hydroxyl radical should also be formed (Reactions 2 and 3).

$$\text{[4Fe-4S]}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{[4Fe-4S]}^{3+} + \text{OH}^- + \text{HO}^+ \quad \text{REACTION 2}$$

$$\text{[4Fe-4S]}^{3+} \rightarrow \text{[3Fe-4S]}^+ + \text{Fe}^{2+} \quad \text{REACTION 3}$$

Hydroxyl radicals react with most organic biomolecules, including amino acids, at nearly diffusion-limited rates; therefore, if one were formed within the active site of a dehydratase, the likely consequence would be the direct oxidation of the protein. However, we were able to quantitatively reac-

FIGURE 4. H$_2$O$_2$ inactivates other [4Fe-4S] dehydratases. A, 6-phosphogluconate dehydratase; B, fumarase A; C, fumarase B. Lysates were prepared from anaerobic cultures of strains LC106, SJ37, and SJ20, respectively, and the indicated concentrations of H$_2$O$_2$ were added for 5 min. Catalase was added to terminate the stress, and residual activity was determined.

FIGURE 5. H$_2$O$_2$ inactivates fumarase A by converting its [4Fe-4S]$^{2+}$ cluster to a [3Fe-4S]$^+$ cluster in vivo. SJ37 cells overproducing fumarase A were harvested at $A_{600} = 0.2$, washed, and resuspended at 1/50th of the original culture volume in 10% glycerol. The resuspended cells were incubated with 200 or 20 μM H$_2$O$_2$ for 1 min at 37 °C. The cell suspension (250 μl) was then transferred into an EPR tube and frozen.

FIGURE 6. The Hpx$^{-}$ mutant is defective in using malate as the sole carbon source. Wild-type (*) and Hpx$^{-}$ (E) cells were cultured in aerobic glucose medium to $A_{600} = 0.15–0.25$. At the arrow, aliquots were removed, washed, and resuspended in aerobic malate medium (wild-type (•) and Hpx$^{-}$ (▫) cells). All media included histidine and aromatic amino acids.
tivate H$_2$O$_2$-treated fumarase (data not shown), which seemed inconsistent with the oxidation of active-site residues. SDS-PAGE analysis of the protein showed that the polypeptide chain was not cleaved. Mass spectroscopy failed to detect any oxidation of histidyl, lysyl, prolyl, or methionyl residues (data not shown).

An alternative model is that a ferryl-like species is generated by the initial electron transfer from the cluster to liganded H$_2$O$_2$ and that the ferryl radical then abstracts a second electron from the cluster instead of releasing a hydroxyl radical (Reactions 4 and 5).

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

$$[\text{4Fe-4S/O}]^{2+} + \text{H}^+ \rightarrow [\text{3Fe-4S}]^{+} + \text{Fe}^{3+} + \text{OH}^-$$  
**REACTION 5**

To test this possibility, we used desferrioxamine and dipyridyl to quantify the release of total iron and ferrous iron, respectively. Ferrous iron is generated by the first mechanistic scheme (Reaction 3), but not by the second (Reaction 6). Although we detected stoichiometric release of one iron atom/cluster (Fig. 8), none of this iron was in the ferrous form (<0.1 atom/cluster) (data not shown). From this result and from the absence of polypeptide oxidation, we infer that a free hydroxyl radical is not generated.

Previous experiments that were conducted with millimolar doses of oxidant indicated that, with time, the cluster disintegration might continue past the [3Fe-4S]$^{+}$ state (19). However, the ease with which we were able to chemically reanimate damaged dehydratases was inconsistent with the formation of apoprotein, which was only slowly reactivated. In fact, EPR analysis showed that the [3Fe-4S]$^{+}$ fumarase cluster was unaffected by a 10-min exposure to 100 μM H$_2$O$_2$ (Fig. 8A). We conclude that, over this period, physiological doses of H$_2$O$_2$ do not directly degrade clusters beyond the easily repaired [3Fe-4S]$^{+}$ state.

**DISCUSSION**

The experiments reported here reveal that submicromolar concentrations of H$_2$O$_2$ are sufficient to destroy enzymatic iron-sulfur clusters. Multiple catabolic and biosynthetic pathways are thereby disrupted. Earlier work with the same strain demonstrated that endogenous H$_2$O$_2$ also reacts with unincorporated iron to produce high levels of DNA damage (8). The two phenotypes are of a piece in that both result from Fenton-type reactions.

The sensitivity of iron-sulfur clusters to univalent oxidants has been appreciated for some time; in fact, much of the toxicity of superoxide is due to inactivation of the same enzymes that...
were identified in this study. Damage by millimolar doses of H₂O₂ has been reported (17, 30). However, although the chemistry is not unexpected, the surprise is that these reactions occur so rapidly. The rate constant of the Fenton reaction was measured to be 76 M⁻¹ s⁻¹ at pH 3, and this number has been cited widely (6). It carried with it the implication that reactions between iron and H₂O₂ are too slow to occur at an important rate in most biological scenarios, where H₂O₂ concentrations are micromolar or lower. However, more recent measurements (8) have shown that, at physiological pH, the rate constant for hexa-aqueous iron is actually orders of magnitude higher, presumably because, at neutral pH, hydroxide anion coordinates iron and lowers its reduction potential. Extrapolating from measurements made at lower temperatures (8), we estimate that the rate may be 20,000–30,000 M⁻¹ s⁻¹ at 37 °C. The same enhancement evidently pertains to the substrate-binding iron atom within a dehydratase iron-sulfur cluster.

The biological consequence of this high reactivity is that far less H₂O₂ is needed to create toxicity than had been suspected. The data directly show that E. coli must synthesize scavenging enzymes to avoid being poisoned by the high nanomolar H₂O₂ that it makes through the adventitious oxidation of its own redox enzymes. Basal levels of the NADH peroxidase solve that problem. However, this basic vulnerability is still exploited by phagocytes and plants, both of which suppress the growth of invading bacteria by generating far higher levels of H₂O₂ either directly or through the synthesis of redox-active antibiotics (31–34).

These deliberate oxidative assaults, as well as the H₂O₂ that accumulates in the environment through chemical oxidation processes, are a true threat to bacteria. Because H₂O₂ influx across membranes can outstrip the action of scavenging enzymes, extracellular concentrations of 5 μM H₂O₂ are sufficient to raise the intracellular H₂O₂ in wild-type (scavenger-proficient) cells to ~1 μM (2). Therefore, as little as 5 μM environmental H₂O₂ is sufficient to cause the enzyme inactivity and pathway defects that were reported in this study.

Interestingly, lactic acid bacteria employ a similar stratagem to inhibit competing organisms, utilizing lactate and pyruvate oxidases, which can drive H₂O₂ levels in their immediate environment up to millimolar levels (35, 36). How do the lactic acid bacteria themselves tolerate the stress that they are creating? Lactic acid bacteria do not rely upon pathways that contain iron-sulfur dehydratases. Instead, they obtain from their environment the amino acids that dehydratase-dependent biosynthetic pathways would normally produce, and they ferment sugars to lactic acid rather than using the tricarboxylic acid cycle.

To defend themselves against such H₂O₂-mediated assaults, microbes throughout the biotic world have evolved H₂O₂-inducible defenses, most of which are either homologs or analogs of the OxyR system of E. coli. The frontline defense of these systems is the strong induction of scavenging enzymes, but the repair of damaged clusters is also an important feature. Ongoing cluster repair may contribute to the disparity between the rates of enzyme damage that we measured in vitro and the apparent rates that we determined in vivo. In a previous study (19), we observed that cells quantitatively repaired H₂O₂-damaged dehydratases, but we were perplexed as to why the hydroxyl radical, which presumably had been formed upon cluster oxidation, did not irreversibly damage active-site amino acid residues. This puzzle is resolved by the observation that ferric (rather than ferrous) iron is released during cluster oxidation. Therefore, a ferryl radical (or a non-diffusing hydroxyl radical) evidently pulls a second electron from the residual cluster rather than allowing an unexpended hydroxyl radical to be released into the active-site bulk solution. Interestingly, an analogous phenomenon was noted for the PerR protein of Bacillus subtilis in that a Fenton reaction between its prosthetic ferrous iron atom and H₂O₂ caused immediate oxidation of the iron ligands rather than the more widely distributed oxidation of the polypeptide, as might have been expected from a diffusible hydroxyl radical (37).

In the dehydratase case, we do not stipulate whether the second electron transfer would generate an intermediate all-ferric [4Fe-4S]³⁺ cluster or whether it would create a disulfide bond concomitant with ferric iron release. However, in either case, polypeptide oxidation would be avoided. The physiological significance is that the enzyme can be restored to its native state by accelerating the rate at which H₂O₂ produces DNA damage (39–41). The OxyR system addresses this threat by inducing the synthesis of Dps, a ferritin-like storage protein that scavenges loose iron and sequesters it into an unreactive ferric hydroxide core (8, 42–44). Thus, it has become clear that several aspects of the inducible defense against H₂O₂ are well matched to the threat that H₂O₂ poses.

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