Iron enzyme ribulose-5-phosphate 3-epimerase in *Escherichia coli* is rapidly damaged by hydrogen peroxide but can be protected by manganese

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H_2O_2 is commonly generated in biological habitats by environmental chemistry and by cellular immune responses. H_2O_2 penetrates cells, disrupts metabolism, and blocks growth; it therefore is of interest to identify the major cellular molecules that H_2O_2 damages and the strategies by which cells protect themselves from it. We used a strain of *Escherichia coli* that lacks catalases and peroxidases to impose protracted low-grade H_2O_2 stress. Physiological analysis indicated that the pentose–phosphate pathway, in particular, was poisoned by submicromolar intracellular H_2O_2. Assays determined that ribulose-5-phosphate 3-epimerase (Rpe) was specifically inactivated. In vitro studies demonstrated that Rpe employs a ferrous iron atom as a solvent-exposed cofactor and that H_2O_2 rapidly oxidizes this metal in a Fenton reaction. The oxidized iron is released immediately, causing a loss of activity. Most Rpe proteins could be reactivated by remetallation; however, a small fraction of proteins were irreversibly damaged by each oxidation cycle, and so repeated cycles of oxidation and remetallation progressively led to permanent inactivation of the entire Rpe pool. Manganese import and iron sequestration are key elements of the H_2O_2 stress response, and we found that manganese can activate Rpe in vitro in place of iron, converting the enzyme to a form that is unaffected by H_2O_2. Indeed, the provision of manganese to H_2O_2–stressed cells protected Rpe and enabled the pentose–phosphate pathway to retain function. These data indicate that mononuclear iron enzymes can be primary targets of H_2O_2 stress and that cells adapt by shifting from iron- to manganese-centered metabolism.

hydroxyl radical | MntH | oxidative damage | OxyR

Catalases and peroxidases were among the first enzymes discovered, and their ubiquity among the biota prompted biologists to speculate that H_2O_2 must be a common by-product of the aerobic metabolism and that, if not scavenged, it must critically harm cells (1). Since then, workers have recognized that H_2O_2 also is generated by extracellular processes, including photochemical processes in natural waters and the autoxidation of S and metal species at interfaces between anoxic and oxic sediments. Perhaps more critically, H_2O_2 is generated by the antimicrobial responses of both plant and mammalian higher organisms as a strategy to stave off infection (2, 3). Local H_2O_2 concentrations at plant wound sites and in macrophages rise to micromolar levels, which are sufficient to suppress microbial growth.

Virtually all microbes engage specialized stress responses to fend off exogenous H_2O_2. Among the bacteria, the OxyR and PerR systems are widespread (4, 5). OxyR, which has been closely studied in enterics, is a transcription factor that is activated when H_2O_2 oxidizes a key cysteine residue; the subsequent formation of a disulfide bond locks the protein into an active form that stimulates the transcription of *ca.* two dozen genes (6, 7). Mutants that lack OxyR grow poorly or not at all in environments that contain H_2O_2.

Still, fundamental aspects of H_2O_2 stress remain poorly understood. Perhaps most importantly, the primary intracellular targets of H_2O_2 have not been identified fully. A technical difficulty has hindered work on this problem: When laboratory cultures are stressed with micromolar H_2O_2, growth stops briefly, but the H_2O_2 is scavenged quickly, and growth resumes. Under these circumstances it has been difficult to identify the specific cellular processes and biomolecules that are affected. One way around this problem has been the use of strains that lack scavenging enzymes (8). *Escherichia coli* catalase/peroxidase mutants (*katG katE ahpCF*, denoted Hpx^-) grow at wild-type rates in anaerobic cultures. When inoculated into aerobic medium, H_2O_2 accumulates to *ca.* 1 micromolar, which equilibrates between the cytoplasm and the extracellular environment. This concentration exceeds the threshold that activates OxyR, and indeed Hpx^- *ΔoxyR* mutants die quickly in aerobic medium (9). Thus, the Hpx^- strains offer the opportunity to identify the primary biomolecules that H_2O_2 damages as well as the key defensive strategies that OxyR triggers.

Thus far it has become clear that H_2O_2 damages DNA through standard Fenton chemistry:

$$Fe^{2+} + H_2O_2 \rightarrow [FeO^{2+}] + H_2O \quad [1]$$

$$[FeO^{2+}] + H^+ \rightarrow Fe^{3+} + HO \quad [2]$$

The Fe that is involved belongs to the cellular pool of unincorporated Fe, some of which presumably associates with nucleic acids (10). A ferryl radical (FeO^{2+}) is the immediate product, but ultimately a hydroxyl radical is released that can attack directly both the sugar and base moieties of DNA (11). Intracellular H_2O_2 also disables a family of dehydratases that contain [4Fe-4S] clusters; the chemistry is similar, in that H_2O_2 directly oxidizes a catalytic Fe atom that is exposed to solvent (12). The oxidized cluster is unstable, the key Fe atom dissociates, and enzyme activity is lost. These enzymes were first shown to be oxidative targets in studies of superoxide (13–17). Exacerbating this situation, H_2O_2 also inhibits the cellular Fe–S cluster assembly machinery, probably by oxidizing nascent clusters as they are assembled on the IscU scaffold protein (18). Finally, H_2O_2 has been shown to disrupt the Fur Fe-sensing system (19). In this case the proximate cause has not been demonstrated, but it seems likely that H_2O_2 oxidizes the Fe^{2+} atom that normally binds to the Fur transcription factor.

The OxyR regulon stimulates the synthesis of several proteins whose roles are well-suited to addressing these effects. The HPI catalase and AhpCF peroxidase are induced to drive back down the intracellular H_2O_2 level (7). The Dps Fe-storage protein sequesters unincorporated Fe and thereby diminishes the amount

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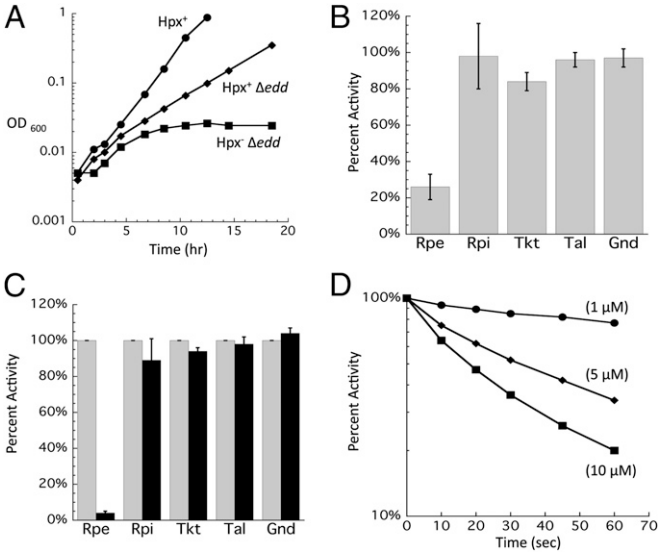

Fig. 1. (*A*) Hpx⁻ Δ*edd* cells cannot grow on gluconate because of damage to Rpe. After anaerobic preculture, cells were diluted into aerobic minimal gluconate medium at time 0. Data presented are representative of multiple experiments. (*B*) Activities measured in extracts prepared from Hpx⁻ Δ*edd* cells grown aerobically. Values were normalized to those of anaerobic cells. Gnd, 6-phosphogluconate dehydrogenase; Rpi, ribose-5-phosphate isomerase; Tal, transaldolase; Tkt, transketolase. (*C*) Activities measured before (gray bars) and after (black bars) 10 μM H_2O_2 was added to cell extracts prepared from anaerobically grown Hpx⁻ cells. Error bars represent SD from the mean of three independent experiments. (*D*) Time course of the inactivation at 0 °C of Rpe in cell extracts treated with the indicated concentrations of H_2O_2. Extracts were prepared from anaerobically grown Hpx⁻ cells.

of DNA damage (9, 20–22). An alternative Fe–S assembly machine, the Suf system, is resistant to H_2O_2 and is induced to compensate for the failure of the Isc system (18). The Fur regulator is induced to higher levels as a hedge against its lower efficiency (19, 23).

Recently we found that the induction of an Mn import system also is critical, because Hpx⁻ Δ*mntH* mutants stop growing when they are aerated (24). *E. coli* normally does not import a significant amount of Mn. We speculated that *E. coli* might use ferrous Fe as a cofactor in nonredox enzymes, which would place such enzymes at risk during periods of H_2O_2 stress. Induction of the MntH transporter might enable Mn to replace Fe in these enzymes; because Mn does not react with H_2O_2, the enzymes might be protected.

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

Results

Ribulose-5-Phosphate 3-Epimerase Is Vulnerable to H_2O_2 Damage in Vivo. Strains of *E. coli* that cannot scavenge H_2O_2 gradually accumulate *ca*. 1 micromolar H_2O_2 (8). This level moderately exceeds the dose that activates the OxyR response, and so it is likely that these strains confront protracted H_2O_2 stress of a magnitude similar to that found in some natural habitats. Growth studies of this strain have shown that most catabolic and biosynthetic pathways continue to function, as is consistent with the observation that this level of H_2O_2 is innocuous to the vast majority of enzymes. However, in the process of testing the H_2O_2 sensitivity of central metabolic pathways, we noted that an Hpx⁻ Δ*edd* mutant is unable to grow in aerobic gluconate medium (Fig. 1*A*). This strain lacks 6-phosphogluconate dehydratase and therefore requires gluconate flux through the pentose–phosphate pathway (Fig. 2). The cells began to lag when the H_2O_2 concentration had reached ~0.8 μM (Fig. S1), indicating that very low levels of H_2O_2 can inactivate this pathway.

The fact that Hpx⁻ *edd*⁺ cells can grow on gluconate via the alternative Entner–Doudoroff pathway implied that the block must occur downstream of gluconate import. The five pentose–phosphate pathway enzymes were assayed in extracts that had been prepared from aerobic cultures of wild-type and Hpx⁻ strains. No differences were found in the levels of 6-phosphogluconate dehydrogenase, ribose-5-phosphate isomerase, transketolase, and transaldolase (Fig. 1*B*). However, ribulose 5-phosphate 3-epimerase (Rpe) showed low activity. To determine whether H_2O_2 can inactivate Rpe directly, Hpx⁻ cells were grown anaerobically, cell extracts were prepared, and the extracts then were briefly challenged with H_2O_2 in vitro. Rpe rapidly lost activity (Fig. 1*C*). Even at 0 °C, 5 μM H_2O_2 inactivated most of the enzyme within 1 min (Fig. 1*D*); the measured rate constant for inactivation (4,000 M^{-1} s^{-1}) approximated those of H_2O_2-sensitive Fe/S dehydratases (12).

Rpe Can Be Metallated by Various Divalent Metals That Result in Variable Properties. To study Rpe in vitro, Rpe from *E. coli* was purified using a His tag, which was removed after purification. Rpe is known to require a divalent metal for activity, but the identity of the metal used in vivo remained uncertain (25). A previous study of Rpe from *Streptococcus pyogenes* reported that it copurified with substantial Zn^{2+}, leading the authors to speculate that this is its physiological metal. Akana et al. (25) reported that Mn^{2+} and Co^{2+} also would activate Rpe in vitro, but Mg^{2+} and Fe^{2+} would not. However, these experiments were conducted under aerobic conditions, and the Fe^{2+} probably oxidized to Fe^{3+}. We used chelators to remove any associated metals from the purified Rpe from *E. coli*, and we then tested various metals for their ability to activate the enzyme in vitro under anaerobic conditions. Of the nine metals used, Ca^{2+}, Cd^{2+},

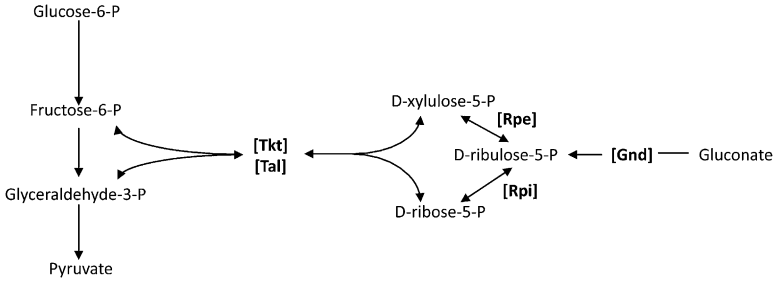

Fig. 2. The pentose–phosphate pathway. Relevant intermediates are listed, with key enzymes denoted in brackets. Arrows indicate direction of each reaction.

Table 1. Properties of various Rpe metalloforms

Metal	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})	$t_{1/2}$ of metal dissociation
Fe^{2+}	67,000 ± 7,700	1.6 ± 0.2	4.2×10^7	50 min
Mn^{2+}	30,400 ± 1,700	1.8 ± 0.1	1.7×10^7	3.5 min
Co^{2+}	14,700 ± 900	2.4 ± 0.4	0.6×10^7	20 h
Zn^{2+}	1,300 ± 100	4.8 ± 0.5	0.02×10^7	8 h

k_{cat} and K_M values were determined as described in *Materials and Methods*. Error represents SD from the mean of three independent measurements. Dissociation rates were determined by measuring the loss of Rpe activity during incubation of metal-loaded enzyme with an excess of the chelator DTPA at 23 °C. DTPA was shown to trap dissociated metals but not to extract them actively (*SI Materials and Methods*). Measurements represent the averages of three independent experiments.

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

The kinetic constants of the four metalloforms of Rpe were determined (Table 1). Strikingly, the Zn^{2+} metalloform had a much lower k_{cat} and higher K_M than the other metalloforms; thus the catalytic efficiency (k_{cat}/K_M) of the Zn^{2+} metalloform was much worse than those of the Mn^{2+}, Fe^{2+}, and Co^{2+} forms, which were roughly comparable. The dissociation rates for each of the activating metals were measured also (Table 1). Despite providing the most catalytically efficient metalloform, Mn^{2+} rapidly dissociated from Rpe, but the Fe^{2+} metalloform was moderately more stable. Interestingly, Zn^{2+} bound tightly to the enzyme.

Of these four metals, only Fe^{2+} and Zn^{2+} are found in *E. coli* in substantial concentrations under normal growth conditions. *E. coli* lacks a Co^{2+} importer, and Mn^{2+} is not actively imported during routine growth (24). It seemed likely that Fe^{2+} is the metal that provides activity to Rpe under normal physiological conditions.

Fenton Chemistry Is Responsible for the Inactivation of Rpe. The proposed mechanism of Rpe implies that the metal will be exposed to solutes, including H_2O_2 (Fig. 3); therefore, we tested whether Fenton chemistry was responsible for loss of Rpe activity. The sensitivity to H_2O_2 of each of the metalloforms of Rpe was tested. When purified Rpe was loaded with Mn^{2+}, Co^{2+}, or Zn^{2+}, there was no loss in activity when challenged with H_2O_2 (Fig. 4). However, when Rpe was metallated with Fe^{2+}, the activity was quickly lost. Because the enzyme that is found in cell extracts is acutely sensitive to H_2O_2, we concluded that Fe^{2+} is indeed the native cofactor that provides activity in laboratory cultures.

Reactions between H_2O_2 and Fe^{2+} initially generate ferryl radicals, which have a substantial lifetime before decomposing into Fe^{3+} and a hydroxyl radical (11, 26). Hydroxyl radicals react at nearly diffusion-limited rates with most biomolecules, including proteins, raising the possibility that H_2O_2 exposure might lead to covalent Rpe damage. We found, however, that when Fe-loaded Rpe was inactivated with H_2O_2, about 80% of the initial Rpe activity could be recovered by adding Fe^{2+} back to the enzyme (Fig. 5*A*). Reductants alone were insufficient, confirming that the oxidized Fe atom had dissociated from the enzyme. Similar results were obtained by the addition of Co^{2+} or Mn^{2+}. Full activity was never recovered. Thus, we concluded that at most only a very minor fraction of the protein was critically and irreversibly damaged; instead, most of the activity loss was caused by the dissociation of Fe.

In vivo experiments showed a similar outcome. When Hpx^- Δ*edd* cells were exposed to a bolus of H_2O_2, activity quickly declined. After the H_2O_2 was removed from the culture, activity rebounded to about 70% of the original activity, even though new protein synthesis was blocked (Fig. 5*B*). The recovery of activity inside the cell reached the same level as when activating metals were added back to the lysate of the H_2O_2-treated cells.

Repeated Rounds of Inactivation Are Required to Damage Rpe Permanently. The data suggested that when Fe-loaded Rpe is oxidized by H_2O_2, the catalytic Fe cofactor is oxidized and lost; however, despite the presumptive formation of a hydroxyl radical [Eqs. **1** and **2**], only a small fraction of those events caused irreversible protein damage. Inactivation was predominantly caused by metal loss. In vivo, however, continual enzyme remetallation should occur. When Rpe activity was tracked during extended Hpx^- growth in aerobic medium, activity quickly fell. At early time points most of the enzyme was in a nonmetallated but reactivatible form, because activity could be recovered by metal addition in vitro. However, the amount of recoverable activity declined progressively, suggesting that Rpe gradually accrued irreversible damage from multiple rounds of Fenton reactions (Fig. 5*C*).

To replicate cycles of damage and remetallation in vitro, purified Rpe was exposed to Fe, ascorbate, and oxygen in vitro. The oxidation of Fe^{2+} by oxygen ensured the constant presence of

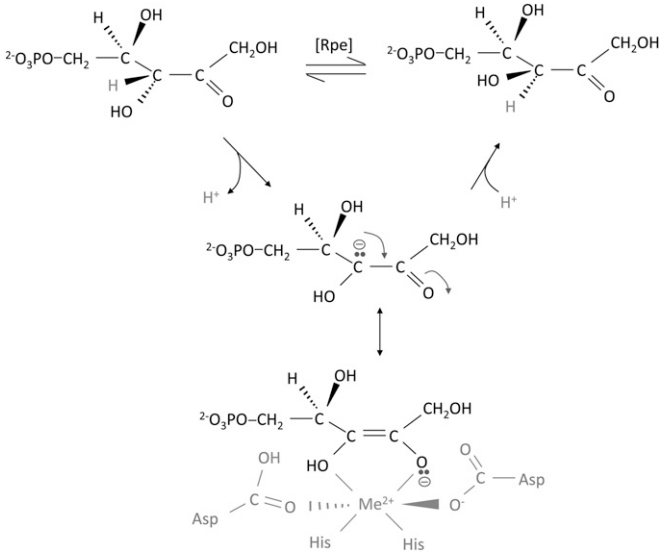

Fig. 3. Rpe active site structure and mechanism. Rpe interconverts ribulose-5-phosphate and xylulose-5-phosphate by abstracting a proton from one face of the C-3 carbon and then adding a proton to the opposite face. The metal coordinates substrate and stabilizes the intermediate oxyanion (25).

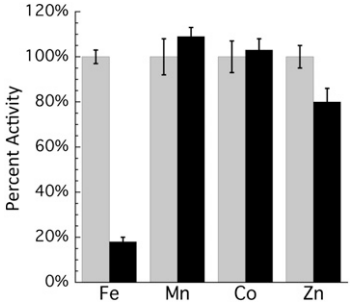

Fig. 4. Sensitivity of Rpe metalloforms to H_2O_2 in vitro. Pure Rpe was metallated with the indicated metals. Activity then was measured before (gray bars) and after (black bars) treatment with 500 μM H_2O_2 for 2 min. Error bars represent SD from the mean of three independent measurements.

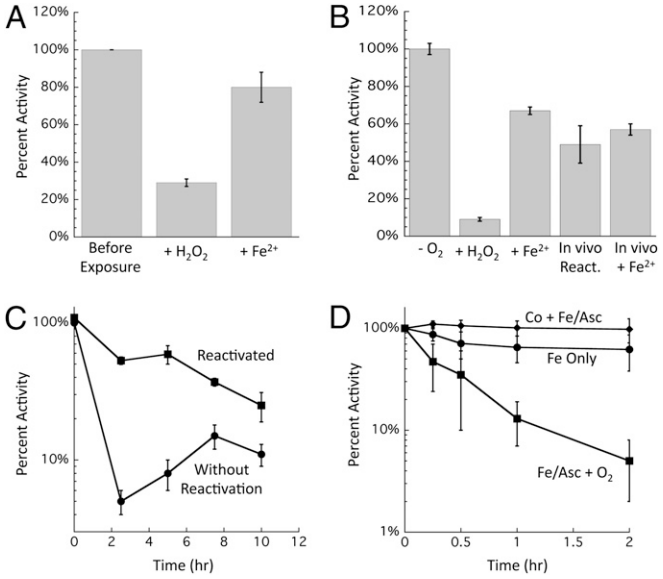

Fig. 5. Reversibility of Rpe inactivation. (*A*) A single cycle of in vitro damage is mostly reversible. Pure Rpe was metallated with 100 μM Fe^{2+} before being treated with 200 μM H_2O_2 for 5 min. Catalase and DTPA were added to remove Fe and H_2O_2, and Rpe was assayed. Damaged enzyme then was reactivated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

a low level of H_2O_2 (27), and the reduction of Fe by ascorbate regenerated Fe^{2+}. At various time points the Fe and H_2O_2 were removed by diethylenetriaminepentaacetic acid (DTPA) and catalase, and the amount of functional Rpe polypeptide was determined by the addition of Co^{2+} before assay. When ascorbate was omitted, activity diminished only at first and thereafter remained constant at about 65% of the starting activity, consistent with a single cycle of enzyme damage. However, when ascorbate was added to enable the cyclical activation and oxidation of the enzyme, activity dropped steadily, down to 15% after 1 h and to about 3% after 2 h (Fig. 5*D*). Taken together, these results indicate that a single bolus of Fenton chemistry irreversibly damages only a fraction of the Rpe molecules. Complete inactivation of the population results from repeated cycles of the reaction.

Mn Protects Rpe from Damage by H_2O_2. The induction of an Mn importer, MntH, has been shown to be a critical aspect of the response of *E. coli* to H_2O_2 (24, 28). We proposed recently that the role of Mn in the protection against H_2O_2 stress might be to replace Fe^{2+} in the mononuclear metal sites of enzymes, thereby diminishing the frequency of Fenton chemistry in those sites. To test this idea with respect to Rpe, Hpx^- Δ*edd* cells were grown aerobically on gluconate with or without Mn supplementation. As shown in Fig. 6*A*, the addition of Mn fully suppressed the aerobic growth phenotype in Hpx^- Δ*edd* cells. To verify that the protective effect was caused by the protection of Rpe, its activity was measured in samples harvested from cultures grown both with and without Mn supplements. Fig. 6*B* shows that the supplements boosted Rpe activity by several-fold at the 10-h time point, when unsupplemented cells began to lag. Because the dissociation rate of Mn in vitro is so fast, these assays almost certainly underrepresented the activity of Rpe containing Mn. Therefore, we sought to quantify the amount of functional Rpe polypeptide by providing Co^{2+} to the extracts. The samples from Mn-supplemented cultures showed nearly 100% activity, compared with less than 25% activity from untreated cultures (Fig. 6*B*). These data support the idea that Mn protects Rpe from H_2O_2 by replacing Fe^{2+} in the active site during H_2O_2 stress.

Fe Must Be Accessible to Solvent for Enzymes to Be Vulnerable to H_2O_2. The full range of enzymes that use Fe^{2+} as a cofactor in vivo—and therefore might be vulnerable to H_2O_2—is unknown. Presumably many enzymes that require divalent metal cofactors might be able to use Fe^{2+}. However, Fenton chemistry requires the direct binding of Fe to H_2O_2, and so we suspected that only those enzymes that might have undercoordinated Fe in their active sites are potentially vulnerable. To test this idea, transketolase was examined. Transketolase requires a divalent metal to form a bridge between the polypeptide and a thiamine pyrophosphate (TPP) cofactor, but the metal does not participate in the reaction chemistry. The metal itself is buried beneath the bound cofactor and is not exposed to solvent (PDB code 1QGD). We determined that Fe^{2+} was able to activate transketolase as efficiently as did Mg^{2+}. However, in contrast to Rpe, the addition of H_2O_2 did not affect the activity of the Fe^{2+}/TPP-loaded enzyme (Fig. 7). These results demonstrate that enzymes that use Fe^{2+} as a metal cofactor are sensitive to H_2O_2 only if the metal site is exposed to solvent; the Fe^{2+} is fully coordinated, Fenton chemistry is blocked, and the enzyme is not vulnerable to H_2O_2. This feature presumably will circumvent damage to many enzymes, such as transketolase, that might occasionally or frequently bind Fe^{2+} as a divalent cofactor.

Discussion

In addition to their facility at redox reactions, transition metals excel at surface chemistry, in which they directly coordinate substrates and thereby activate them for a wide variety of reaction types. Rpe is an example of the latter: Its mononuclear metal

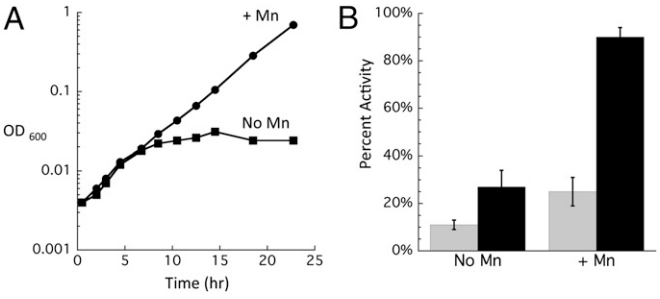

Fig. 6. Mn supplements protect Rpe against irreversible damage in vivo. (*A*) After anaerobic preculture, cells were diluted at time 0 into aerobic minimal gluconate medium with or without 50 μM $MnCl_2$. Data are representative of multiple growth experiments. (*B*) Cells were handled as in *A* except that aerobic inoculum was to 0.05 OD. At various times, aliquots were taken, and Rpe activity was measured before and after reactivation with Co. Time 0 is an anaerobic time point. Error bars represent the SD from the mean of three independent experiments.

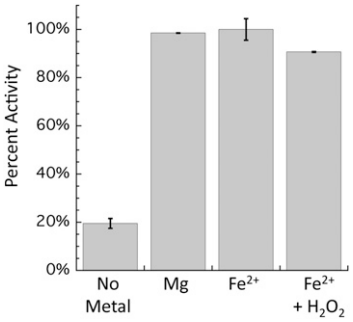

Fig. 7. Transketolase and necessity of solvent exposure for metal sensitivity to chelation and to H_2O_2. Metals were added to pure transketolase (Sigma) before the addition of TPP and 500 μM H_2O_2. After mixing, samples were incubated for 5 min and then assayed as described in *SI Materials and Methods*.

atom binds the carbonyl and carboxylate moieties of ribulose-5-phosphate, facilitating epimerization by stabilizing the deprotonated anionic intermediate. In addition, over the course of the reaction, the metal alternatively coordinates and releases the aspartyl residues that act as proton acceptors and donors to the substrate. Although a nontransition metal such as Mg can suffice in enzymes where its sole role is to neutralize charge, transition metals often are requisite when the metal environment must change during the reaction cycle, because they can tolerate changes in their coordination sphere with minimal activation energy.

Our study indicates that Fe is the transition metal that activates Rpe. This finding makes both ecological and evolutionary sense, because Fe typically is abundant in anaerobic habitats, including both the mammalian gut in which *E. coli* dwells and the anaerobic world in which Rpe originally evolved. Indeed, the service of ferrous Fe in nonredox enzymes probably is systematically underrecognized, because Fe is oxidized quickly and released when these enzymes are studied in aerobic buffers. Thus, although in vitro experiments suggest that Mn can activate scores of enzymes in *E. coli* (http://www.Ecocyc.org/), in reality Mn is scarcely imported under routine growth conditions (24). More likely, Fe is the cognate metal of many of these enzymes.

Fe Enzymes Are Targets of Oxidants. Catalase and peroxidase activities are sufficient to cope with the H_2O_2 that is formed as a by-product of aerobic metabolism, but the diffusion of H_2O_2 into the cell from environmental sources can drive its steady-state levels above the threshold of toxicity (29). Recent studies from several laboratories indicate that protein damage is likely to determine the fate of oxidant-exposed cells (30–33). Rpe, and probably mononuclear Fe enzymes in general, now join Fe–S dehydratases as primary classes of enzymes that H_2O_2 damages. In both cases H_2O_2 directly oxidizes a solvent-exposed Fe atom that normally serves to bind substrate; the oxidized Fe atom then dissociates from the enzyme. Interestingly, the hydroxyl-like oxidants that are formed need not damage the active sites of either type of enzyme fatally—the enzymes can be reactivated repeatedly, both in vivo and in vitro. When H_2O_2 oxidizes Fe–S clusters, hydroxyl radical release is averted when the nascent ferryl radical abstracts a second electron from the cluster (12). We speculate that Rpe similarly might be spared covalent damage because the ferryl radical usually dissociates from the active site and does not release a hydroxyl radical until it enters the bulk solution. Upon reflection, it is probably an essential feature of Fe-containing enzymes that they are not irreversibly damaged upon each encounter with H_2O_2. Even in innocuous habitats wild-type *E. coli* contains about 20 nM steady-state H_2O_2 from endogenous sources (29); because the inactivation rates of these enzymes are likely 10^4–10^5 M^{-1} s^{-1} at physiological temperatures (Fig. 1*D*) (12), they are likely to be oxidized by H_2O_2 on the order of every 10 min. This situation would be intolerable were the polypeptide irreparably damaged each time.

Role of Alternative Metals in Fe Enzymes. The reliance of metabolism upon Fe-cofactored enzymes presents problems during periods of either Fe starvation or oxidative stress. One apparent solution is to replace Fe with Mn. When the problem is Fe scarcity, the deactivation of the Fur repressor stimulates synthesis of MntH, the Mn importer (28). Presumably the resultant Mn influx allows mononuclear enzymes such as Rpe to continue to work. Because Mn does not share the reduction potential of Fe, the solution is slightly more complicated for redox enzymes: Key Fe-dependent enzymes, such as superoxide dismutase and ribonucleotide reductase, are replaced by distinct isozymes that bind and poise Mn at an appropriate potential (34, 35).

The induction of the Mn transporter is not sufficient to protect these enzymes from H_2O_2, however, if the H_2O_2-stressed cell still contains ample Fe that might yet outcompete Mn for enzyme binding. Thus, the induction of MntH by OxyR is supplemented by its induction of the Fur repressor (23), which inhibits expression of Fe importers, and of Dps, which sequesters intracellular Fe in a ferritin-like storage protein (9, 20–22). In collaboration these systems ensure that the Fe/Mn ratio tips toward the latter. Carbonylation assays suggest that many cellular proteins are protected in this way (24).

Although the evidence fits the idea that Mn protects Rpe by integrating as the cofactor metal, we were unable to demonstrate this notion directly: By the point of assay, Mn was not bound to the Rpe that was harvested from these cells. Thus, some uncertainty remains. Still, the rapid dissociation of this weakly bound metal is expected during extract preparation. In any case, it is clear that Mn import blocked enzyme damage and sustained activity. An alternative model for the antioxidant effects of Mn (36–41) is that it acts as a chemical scavenger of reactive oxygen species, including H_2O_2 (37, 39, 42–44). That model seems not to apply to *E. coli*, because direct measurements showed that Mn import did not affect H_2O_2 levels (24). Further, we found that excess Co, which also can activate Rpe but does not chemically degrade H_2O_2, was able to substitute for Mn in preserving the function of the pentose–phosphate pathway during H_2O_2 stress (Fig. S2). It seems possible that this Mn protection scheme operates constitutively in those lactic acid bacteria that produce H_2O_2 in high quantities, because these bacteria characteristically require millimolar levels of intracellular Mn for good growth (45). Work in the Daly laboratory has suggested that additional mechanisms of Mn-based protection also might occur in these organisms (36). It will be interesting to see whether these organisms have evolved enzymes that are better than *E. coli* Rpe at binding Mn, or if the requirement for such high intracellular Mn is a reflection of weak binding in these cells as well.

It has long been of interest to identify the mechanisms that ensure that enzymes bind their cognate metals. The example of *E. coli* experiencing H_2O_2 stress, during which cellular Mn^{2+} rises from 15 to 180 μM (24), indicates that metallation is substantially controlled by the relative concentrations of the competing metals. However, this example also points out that this level of control is not enough, because Zn, which binds more tightly to the Rpe active site than does either Mn or Fe, is abundant inside the cell. Indeed, when Mn-loaded Rpe was coincubated with Zn in vitro, activity steadily declined as the association/dissociation equilibrium of Mn was interrupted by the permanent binding of Zn (Fig. S3). The quick solvation of Mn and Fe from mononuclear enzymes may be the counterpoint to their facility at the exchange reactions that allow them to be good surface catalysts. Inside the cell competition from Zn presumably is suppressed by other intracellular ligands, such as the glutathione pool, that substantially sequester it. Whether the cell also employs systems that either

actively load Fe/Mn into mononuclear enzymes such as Rpe or that facilitate the dissociation of inappropriately bound metals remains to be determined.

Materials and Methods

Reagents, strains, growth conditions, and assays used in this study are described in *SI Materials and Methods*. Strains used in this study are listed in Table S1.

Bacterial cultures were grown aerobically at 37 °C with vigorous shaking or anaerobically at 37 °C in a Coy anaerobic chamber. All enzyme assays were conducted under anaerobic conditions in sealed cuvettetes.

E. coli Rpe was purified by His tag using a variation of the protocol described by Akana et al. (25). Purification details are described in *SI Materials and Methods*. After purification, the His tag was removed. Because EDTA had been included in buffers, the as-isolated enzyme was inactive and recovered activity only upon the provision of metals.

For determinations of kinetic constants, purified Rpe was activated by 500 μM Co, Fe, Mn, or Zn for 10 min before anaerobic assays. Metal dissociation rates were determined by anaerobic incubation of the metal-loaded enzyme with an excess of DTPA; aliquots were withdrawn and assayed at indicated time points. To test sensitivity to H_2O_2, purified Rpe was activated for 5 min in anaerobic buffer containing 100 μM of each metal and then diluted into the Rpe assay mixture. The reaction was run for 6 min, and then H_2O_2 was added (final concentration of 500 μM). Rates were compared before and after H_2O_2 treatment.

Purified transketolase (Sigma) was metallated by 200 μM ferrous ammonium sulfate or $MgCl_2$. After 2 min, TPP was added to a concentration of 2 mM for 2 min before the addition of H_2O_2 (final concentration of 500 μM). Samples then were incubated for an additional 2 min before being assayed.

Complete details for each of the methods described here are given in *SI Materials and Methods*.

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