Inactivation of Dehydratase [4Fe-4S] Clusters and Disruption of Iron Homeostasis upon Cell Exposure to Peroxynitrite*

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Phagocytes produce both nitric oxide and superoxide as components of the oxidative defense against pathogens. Neither molecule is likely at physiological concentrations to kill cells. However, two of their reaction products, hydrogen peroxide and peroxynitrite, are strong oxidants, cell-permeant, and toxic. Hydrogen peroxide generates oxidative DNA damage, while the primary mechanism of toxicity of peroxynitrite has not yet been determined. Recent in vitro studies indicated that peroxynitrite is capable of oxidizing the [4Fe-4S] clusters of a family of dehydratases (Hausladen, A., and Fridovich, I. (1994) J. Biol. Chem. 269, 29405–29408; Castro, L., Rodriguez, M., and RADI, R. (1994) J. Biol. Chem. 269, 29409–29415). We demonstrate here that peroxynitrite at 1% of its lethal dose almost fully inactivated the labile dehydratases in Escherichia coli. The rate at which peroxynitrite inactivated the clusters substantially exceeded the rate at which it oxidized thiols or spontaneously decomposed. These results suggest that these dehydratases may be primary targets of peroxynitrite in vivo. Another consequence of the cluster damage was the release of 100 μM iron into the cytosol. During phagocytosis, this intracellular free iron could increase lethal DNA damage by hydrogen peroxide or protein modification by additional peroxynitrite. In response to peroxynitrite challenges, E.coli rapidly sequestered the intracellular free iron using an undefined scavenging system. The iron-sulfur clusters were more gradually repaired by a process that drew iron from its iron-storage proteins. These are likely to be critical events in the struggle between phagocyte and pathogen.

When phagocytic cells attempt to kill captured pathogens, they utilize specialized enzymes on the interior aspect of the phagosomal membrane to bombard the target cell with superoxide (O$_2^-$) and nitric oxide (NO). The mechanism by which these chemicals damage the invader is unclear. Superoxide cannot penetrate the cytoplasmic membrane of the target cell because of its anionic charge (1). Nitric oxide can cross membranes, but its known direct effects are limited to the reversible inhibition of heme enzymes (2). It therefore appears possible because of its anionic charge (1). Nitric oxide can cross membranes, but its known direct effects are limited to the reversible inhibition of heme enzymes (2). It therefore appears possible that the toxic effects of these species are mediated by their chemical by-products. Superoxide dismutates both spontaneously and enzymatically to form hydrogen peroxide, which is both cell-permeant and a sufficiently strong oxidant to generate lethal DNA damage (for review, see Ref. 3). And nitric oxide reacts with superoxide in the close spaces of the phagosome at a diffusion-limited rate, forming peroxynitrite (ONOO$^-$) (4–7). Peroxynitrite crosses cell membranes and, unlike nitric oxide itself, can rapidly kill cells (8).

The chemistry of peroxynitrite is complex, which makes it difficult to anticipate its primary targets inside the cell. Ground-state peroxynitrite can di valently oxidize nucleophiles, including glutathione and methionine (9, 10); univalently oxidize one-electron donors such as ferrocyanide and ferrocytochrome c (11); and, assisted by transition-metal catalysts, nitrate phenolics such as tyrosine (12, 13). Furthermore, peroxynitrite can form an undefined activated species (commonly denoted HOONO$^+$) that decomposes to nitrite or, alternatively, abstracts electrons from even poor donors, such as deoxyribose (14–16). This oxidant would be expected to have a broad spectrum of potential biological targets. However, reactions between ground-state peroxynitrite and nucleophiles may preclude substantial formation of HOONO$^+$ in the cell.

Because they are both capable univalent oxidants, peroxynitrite might to some extent be expected to resemble superoxide as a toxin. Much of the potency of superoxide arises from its ability to oxidize the [4Fe-4S] clusters of a family of dehydratases that are distributed among both bacterial and mammalian cells (17–22). These cationic clusters serve as Lewis acids during substrate dehydration. However, the transfer of a single electron from the exposed cluster to superoxide destabilizes the cluster, provoking iron loss and inactivation of the enzyme. The primary consequence is that the pathways in which these enzymes function become inoperative, and a secondary effect is that the liberated iron can deposit along the DNA, promoting the generation of mutagenic or lethal DNA damage (23, 24). Ferricyanide, another good univalent oxidant, similarly damages these enzymes in vitro (21). Recently, Hausladen and Fridovich (25) and Castro and Radi (26) determined that peroxynitrite, as well, efficiently inactivates these enzymes in vitro. In those experiments, the damaged enzymes could be reactivated by incubation with ferrous iron and thiols, indicating that peroxynitrite inactivated the enzymes specifically by damaging the [4Fe-4S] clusters (26) (see Reaction 1).

[4Fe–4S]$^{3+}$ + ONOO$^-$ + H$^-$ → [3Fe–4S]$^{3+}$ + HO$^-$ + NO$_2^-$ + Fe$^{3+}$

REACTION 1

The second-order rate constants of the damaging reactions were sufficiently high ($1.4 \times 10^9$ M$^{-1}$ s$^{-1}$) that it is plausible that these enzymes would be primary targets of peroxynitrite in vivo.

The work reported here demonstrates that moderate doses of peroxynitrite do indeed preferentially attack these [4Fe-4S]-containing dehydratases in vivo. Escherichia coli responds to this crisis by rapidly sequestering the free iron and more gradually reactivating the damaged enzymes.

MATERIALS AND METHODS

Chemicals—Sodium nitrite, manganese dioxide, magnesium sulfate heptahydrate, and manganese chloride were purchased from Aldrich.

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Damage to Dehydratase [4Fe-4S] Clusters by Peroxynitrite

1,2,3-Oxidazolidin-5-amino-3-(4-morpholino)-chloride (SIN-1) was from Cayman Chemical Co., Ann Arbor, MI. Deferoxamine mesylate (desferrioxamine), gluconic acid, djenkolic acid, porcine heart isocitrate dehydrogenase, NADP+, NADH, NADPH, NAD+, reduced nicotinamide hypoxanthine dinucleotide (deamin-NADP+), l-malate, EDTA, diethylenetriaminepentaacetic acid (DTPA), sodium sulfite, succinate, 6-phosphogluconate, rabbit muscle lactic dehydrogenase, hydrogen peroxide (H2O2) (30% w/v), ampicillin, isopropyl-β-D-thiogalactopyranoside, nitrophenyl-β-D-galactosidase, nitrate reductase, 4-aminomnitirpine, nitrophosphine, horse heart cytochrome c, xanthine, xanthine oxidase, potassium cyanide, plumbagin, and chloramphenicol were purchased from Sigma. Coomassie protein reagent was from Pierce. β-Mercaptoethanol and sodium citrate were from Fisher Scientific. Water was purified from a Labconco Water Pro system using house deionized water as feedstock.

Peroxynitrite (ONO•O) was synthesized by a procedure suggested by Chris Privale, modified from the original quenched-flow method of Beckman (27). 0.6 M NaNO2 and 0.6 M H₂O₂ in 0.7 M HCl were rapidly mixed in a glass T-junction with outflow to a beaker containing 1.3 M NaOH and manganese dioxide to remove residual hydrogen peroxide. The mixture was then filtered, frozen immediately in a dry-ice ethanol bath, and stored at −80 °C. To determine the yield and purity of peroxynitrite, 10 μl of newly synthesized ONOO− was diluted into 1 N NaOH and scanned spectrophotometrically from 200 to 600 nm. Peroxynitrite concentrations were quantitated by the absorbance at 302 nm. Peroxynitrite concentrations were determined before each experiment and were sometimes further diluted into a 1 N NaOH solution and scanned spectrophotometrically from 200 to 600 nm.

For assay of sulfite reductase, treated cells were resuspended in 0.1 M potassium phosphate buffer (pH 7.8), 5 mM EDTA, and 10 mM MgCl₂ were mixed in a 0.1-ml reaction and allowed to incubate for 5 min at room temperature. The reaction mixture was then diluted into 2 ml of 50 mM Tris (pH 7.65) and boiled for 2 min. Particulates were removed by centrifugation, and 1 ml of the supernatant was assayed for NADH oxidation at 340 nm. For assay of dehydrogenase I, but not NADH dehydrogenase II, can use deamino-NADH as a reductive substrate. Absorbance was monitored at 600 nm.

For assay of dehydrogenase I, extracts were prepared for 6-phosphogluconate dehydrogenase in 50 mM Tris (pH 7.65). The two-step method of Fraenkel was used to determine enzyme activity (34). Extract, 8 mm, 6-phosphogluconate, and 10 mM MgCl₂ were mixed in a 0.1-ml reaction and allowed to incubate for 5 min at room temperature. The reaction mixture was then diluted into 2 ml of 50 mM Tris (pH 7.65) and boiled for 2 min. Particulates were removed by centrifugation, and 1 ml of the supernatant was assayed for 3.3 mm NADH and lactic dehydrogenase.

Extracts were prepared for the assay of fumarase in 50 mM sodium phosphate buffer (pH 7.3). Extracts were assayed in sodium phosphate buffer by the addition of 0.1 ml of 0.5 M l-malate. Absorbance was monitored at 250 nm, with εmax = 1.40 mM −1 cm−1.

For assay of NADH dehydrogenase I, extracts were prepared in 50 mM potassium phosphate buffer (pH 7.8). Extracts were immediately assayed after lysis in a 1-ml reaction containing extract, 50 mM potassium phosphate buffer (pH 7.8), 3.3 mM KCN, 0.4 mM plumbagin, and 0.25 mm deamin-NADPH. NADH dehydrogenase I, but not NADH dehydrogenase II, can use deamino-NADPH as a reductive substrate. Absorbance was monitored at 340 nm.

For assay of NADH dehydrogenase II, lysates were incubated on ice overnight to eliminate NADH dehydrogenase I activity and then assayed for NADH oxidation at 340 nm by the addition of 0.25 mM NADH.

For assay of sulfite reductase, treated cells were resuspended in 0.1 mM potassium phosphate buffer (pH 7.7) and 0.1 ml of EDTA, lysed, and assayed with 0.5 mM sodium sulfite as an electron acceptor. The oxidation of 0.2 mm

The abbreviations used are: deamin-NADH, reduced nicotinamide hypoxanthine dinucleotide; DTPA, diethylenetriaminepentacetic acid; G, guass.
NADPH was monitored (35). β-Galactosidase activity (32), glucose-6-phosphate dehydrogenase (36), fructose-1,6-diphosphate aldolase (37), β-lactate:NAD⁺ oxidoreductase (38), pyruvate dehydrogenase (39), citrate synthase (40), isocitrate dehydrogenase (41), catalase (42), glutathione reductase (43), aldolase (37), fructose-1,6-diphosphate aldolase (37), glyceraldehyde-3-phosphate dehydrogenase (44), glyceroldehyde-3-phosphate dehydrogenase (45), superoxide dismutase (46), and acid-soluble thiols (42) were determined by standard procedures. Protein content was determined by a Coomassie dye-based protein assay by Pierce.

The succinate oxidase activity of inverted vesicles prepared from peroxynitrite-treated cells was measured by monitoring aerobic respiration in a Clark oxygen electrode. Inverted vesicles were produced from peroxynitrite-treated cells was measured by monitoring aerobic respiration, because the vesicles were produced from high-speed centrifugation of cells suspended in buffer. The vesicles were suspended in buffer and centrifuged at high speed. The suspended vesicles were then incubated with peroxynitrite for 15 s. Desferrioxamine was immediately added after centrifugation to a final concentration of 2 mM, and the suspension was incubated at 37 °C for 15 min. Cells were then centrifuged, washed in 20 mM Tris (pH 7.4), and centrifuged again to pellet. Cell pellets were resuspended in a final volume of 200 μl of 20 mM Tris (pH 7.4), 10% glycerol. 200 μl were then transferred to a 3-mm quartz EPR tube, frozen in dry ice, and stored at −80 °C until assayed.

The EPR signals were measured with a Varian Century series E-112 X-band spectrometer equipped with a Varian TE102 cavity. A liquid nitrogen finger Dewar in which samples were immersed in liquid nitrogen was used to ensure constant temperature from sample to sample. Parameters remained constant between experiments and were as follows: temperature = −125 °C, field center = 1520 G, field sweep = 500 G, modulation amplitude = 12.5 G, receiver gain = 25,000, frequency = 27 GHz, and power = 30 milliwatts. One thousand and five points were collected per scan, and four scans were averaged per sample. Data were analyzed using software from Scientific Software Services, Bloomington, IL. Iron levels were quantitated by normalizing the intensity of the EPR signal to an iron standard sample assayed on the same day. Internal iron concentrations were calculated using the intracellular volume (47). Experiments were repeated a minimum of three times, with error not exceeding 15% between independent experiments and scan profiles exhibiting the same trends.

Experiments that measure the rate of free-iron clearance from a treated cell require that desferrioxamine penetrate the cell and capture free iron quickly. Parallel experiments demonstrated that the addition of 2 mM desferrioxamine protects eukaryotic E. coli from an external bolus of 2.5 mM H₂O₂ in less than 1 min of application. Since desferrioxamine prevents oxidative DNA damage by chelating intracellular free iron (48), we conclude that the addition of 2 mM desferrioxamine penetrates the cell sufficiently rapidly to capture the majority of free iron within the interval of the EPR measurements reported in this study.

RESULTS

Peroxynitrite Specifically Inactivates Dehydratases Containing [4Fe-4S] Clusters—The dilution of alkaline peroxynitrite into neutral medium results in its rapid decomposition, with a half-life of approximately 2 s (14). Thus, the addition of peroxynitrite to suspended cells comprises a bolus of short duration. Pilot experiments demonstrated that while components of growth medium protected growing cultures of E. coli from the toxic effects of peroxynitrite, presumably by reacting with and detoxifying the peroxynitrite, cells suspended in buffer were rapidly inactivated. Concentrations of peroxynitrite in excess of 1 mM were lethal to 99% of the cell population, but virtually all cells (>90%) survived exposure to boluses of less than 500 μM. When nutrients were restored to the cells immediately after peroxynitrite exposure, it was observed that, whereas sham-treated cells resumed growth immediately, those that had been exposed to doses of 100 μM peroxynitrite exhibited a 10-min growth lag (Fig. 1). No lag occurred when the peroxynitrite was neutralized prior to its addition to the cells, confirming that the growth inhibition was a consequence of damage done by peroxynitrite per se rather than by its decomposition products. We were interested in defining the molecular damage produced by these moderate doses of peroxynitrite.

Acotatase, 6-phosphogluconate dehydratase, and fumarase A are members of the small family of hydrolyases in E. coli that employ [4Fe-4S] clusters to catalyze the dehydration of their substrates. These enzymes were extensively inactivated when cells were exposed to a pulse of even 10 μM peroxynitrite (Table I, Fig. 2). This inactivation did not involve partially reduced oxygen species, since the enzymes were also inactivated when cells were exposed to peroxynitrite anaerobically (not shown). In contrast, all but 1 of the 16 other enzymes that were sampled were unaffected by even 100 μM doses, including several that utilize iron-sulfur clusters and hemes as redox moieties. These include sulfite reductase, which has a single [4Fe-4S] cluster and a siroheme; succinate dehydrogenase, which contains essential [2Fe-2S], [3Fe-4S], and [4Fe-4S] clusters, and a b-type cytochrome; NADH dehydrogenase I, which has at least two [2Fe-2S] and three [4Fe-4S] clusters; and cytochrome o oxidase, which contains b- and o-type cytochromes. Although E. coli superoxide dismutases can be inactivated by exposure to peroxynitrite in vitro (12), they were not significantly affected in vivo by the doses that inactivated the dehydratases.

Peroxynitrite is a known oxidant of thiols, and about half the soluble thiols were oxidized by 100 μM concentrations. Glyceraldehyde-3-phosphate dehydrogenase of E. coli contains an essential active-site thiol (49, 50). Rabbit glyceraldehyde-3-phosphate dehydrogenase can be inactivated by peroxynitrite in vitro (51). This enzyme lost substantial activity when cells were exposed to 100 μM peroxynitrite, although less so than did the dehydratases. This trend follows the relative rate constants for reactions of peroxynitrite with labile clusters and thiols, as determined in vitro with pig heart mammalian aconitase and cysteine (26, 9). Evidently, the dehydratases are highly specific targets of peroxynitrite in vivo as well.

The Damaged Dehydratases Remain in a Form That Can Be Reactivated in Vivo—A previous study demonstrated that dehydratases damaged by peroxynitrite in vitro could be reactivated by incubation with ferrous iron and sulfide (26). Those results indicated that the inactivity was a consequence of partial destruction of the catalytic [4Fe-4S] cluster. To determine
Peroxynitrite had no detectable effect upon 11 enzyme activities that are not shown: glucose-6-phosphate dehydrogenase, fructose-1,6-diphosphate aldolase, β-lactate:NAD⁺ oxidoreductase, pyruvate dehydrogenase, citrate synthase, isocitrate dehydrogenase, NADH dehydrogenase II, catalase, glutathione reductase, alkaline phosphatase, and β-galactosidase. Cells were grown and assayed for enzyme activity as indicated under “Materials and Methods.”

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Untreated</th>
<th>+ONOOH (% activity)</th>
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</thead>
<tbody>
<tr>
<td>6-Phosphogluconate dehydratase</td>
<td>70</td>
<td>8.5 (12)</td>
</tr>
<tr>
<td>Aconitaseα</td>
<td>124</td>
<td>29 (23)</td>
</tr>
<tr>
<td>Fumarase A</td>
<td>393</td>
<td>113 (29)</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate</td>
<td>243</td>
<td>106 (44)</td>
</tr>
<tr>
<td>dehydrogenase</td>
<td>20</td>
<td>23 (117)</td>
</tr>
<tr>
<td>Succinate oxidase</td>
<td>34</td>
<td>31 (91)</td>
</tr>
<tr>
<td>Sulfite reductase</td>
<td>350</td>
<td>350 (100)</td>
</tr>
<tr>
<td>NADH dehydrogenase I</td>
<td>7300</td>
<td>7600 (104)</td>
</tr>
<tr>
<td>Superoxide dismutaseβ</td>
<td></td>
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α Sum of aconitase activity from two isozymes (71).
β Sum of activity from MnSOD and FeSOD.

Iron Import and Iron-storage Proteins Are Needed for Optimal Reactivation of the Dehydratases—The reconstruction of oxidatively damaged [4Fe-4S] clusters is thought to be an enzymatic process requiring the donation of iron (52, 53). Iron storage proteins are a potential source of the iron needed for repair of the clusters. *E. coli* has two iron storage proteins, bacterioferritin (encoded by bfr) and ferritin (ftrn) (54, 55). The reactivation of 6-phosphogluconate dehydratase was monitored in mutants lacking the internal iron-storage proteins ferritin and bacterioferritin. These strains synthesize normal levels of active dehydratases during routine growth. However, 10 min after peroxynitrite exposure, these strains had recovered only 10–15% of their initial activity, in contrast to the much more efficient recovery of wild-type cells (Fig. 4).

Iron transport into the cell from the external medium is an alternative source of iron for cluster re-assembly. To test this possibility, DTPA, a cell-impermeable iron chelator, was added to the cell suspension immediately after peroxynitrite challenge to block iron uptake. Cells treated in this way regained only 25–30% of enzymatic activity in 10 min, and iron-storage mutants that were treated with DTPA recovered only 5%. It appears that both internal iron reserves and transport from the external medium are needed to supply sufficient iron to rapidly rebuild these clusters. Interestingly, both the ferritin and bacterioferritin proteins appear to be required, raising the possibility that they function in series in an iron-donation pathway. Although iron availability appeared to limit the efficiency of cluster repair in these experiments, the peroxynitrite-challenged cells did not induce the Fur regulon, which directs the synthesis of several iron-uptake systems (31) (data not shown).

**Peroxynitrite Increases the Intracellular Free Iron Concentration in the Cytoplasm**—The requirement for iron to reactivate the dehydratases indicated that iron was released from the clusters when they were damaged by peroxynitrite. The amount of loosely bound iron in the cytoplasm of exponentially growing *E. coli* has been measured by EPR spectroscopy to be approximately 15 µM (24). However, when cells are stressed by superoxide, the oxidation of dehydratase clusters releases sufficient iron that the amount of free iron in the cytosol increases to about 80 µM. A similar increase in free iron occurred after exposure to peroxynitrite (Fig. 5). The iron release was close to maximal after challenge with 100 µM peroxynitrite, in accordance with the sensitivity of the dehydratases (data not shown).

That maximum exceeds by only a small amount the free iron released by superoxide stress. Indeed, when an SOD mutant was challenged with peroxynitrite, little additional iron was released (Fig. 6). These results indicate that superoxide and...
peroxynitrite release iron exclusively from the same set of vulnerable dehydratases. If only one iron atom is lost per cluster, the amount of liberated iron suggests that in *E. coli* the sum concentration of these labile clusters is approximately 100 μM. The plausibility of this number can be checked by calculating the concentrations of the labile dehydratases. Using the turnover number for fumarase A (56) and our measurements of its activity in crude extracts, we estimate that the fumarase A concentration in these cells was 5–8 μM, which represents 10–15 μM [4Fe-4S] clusters, since the enzyme is dimeric. Turnover numbers are not available to make similar calculations for aconitase and 6-phosphogluconate dehydratase. However, a large substrate flux passes through these enzymes in this medium, so it is at least plausible that they are abundant and that there is a total of 100 μM labile clusters in the cell. It is also possible that the enzyme concentration is somewhat lower than this, but that more than one iron atom eventually leave the damaged clusters.

Iron Released from Damaged Dehydratases Is Rapidly Sequestered—The rate of oxidative DNA damage in cells is dictated in part by the amount of free intracellular iron (57, 24), since iron that is adventitiously bound to DNA catalyzes the production from hydrogen peroxide of the damaging hydroxyl radical (58, 48). Superoxide-stressed cells are especially vulnerable to DNA damage because of the large amount of free iron in their cytoplasm (24). We were therefore surprised to observe that peroxynitrite-treated cells were not unusually sensitive to DNA damage when they were subsequently exposed to hydrogen peroxide for 10 min (data not shown). In part, this resistance may have been due to their temporary starvation during the peroxynitrite treatment, since cells must be metabolically active to suffer oxidative DNA damage (33). However, a more important factor may be that the iron released by the peroxynitrite treatment was rapidly sequestered (Fig. 7). The majority of the iron became chelator-inaccessible within 30 s. The high sensitivity to hydrogen peroxide of superoxide-stressed cells may be a result of the continuous turnover of iron from the iron-sulfur clusters, whereas in the experiments reported here iron was liberated by a single pulse of peroxynitrite and could be rapidly retrieved. We attempted to use SIN-1 to continually generate peroxynitrite in cell cultures, so that the free-iron content and peroxide sensitivity of unstarved cells could be assayed simultaneously, but components of the medium scavenged the oxidant and prevented cell damage.

The cytoplasmic iron was cleared much more rapidly than the dehydratases were reactivated, making it unlikely that it simply re-entered the clusters. Mutants that lacked the iron-storage proteins exhibited a slightly but consistently slower rate of iron clearance, suggesting that in wild-type cells some iron may have been deposited in these reservoirs (Fig. 7). However, free iron was still removed rapidly from the cytoplasms of the storage mutants.
The in vitro measurements of Radi et al. (9, 26) indicate that peroxynitrite oxidizes thiols at a 50-fold lower rate than it attacks [4Fe-4S] clusters. In E. coli, thiol concentrations are approximately 2 nm (42), whereas the release of 100 μM iron by peroxynitrite indicates that the cell may contain about 100 μM labile dehydratases. It follows that a molecule of peroxynitrite may be 2.5 times as likely to inactivate a dehydratase as to be scavenged by a thiol. Although other unknown targets may exist, the unique requirements of ground-state peroxynitrite as an oxidant suggest that there may be very few. Because of this high specificity, the toxicity of peroxynitrite, like that of superoxide, may be narrowly directed at a handful of enzymes. It might therefore have even a larger physiological impact than an equivalent amount of a more reactive species whose effects are more broadly distributed among a variety of target molecules.

Peroxynitrite decomposes spontaneously with a half-life of approximately 2 s in the neutral pH range that pertains in vivo. Although this limits its efficacy in vitro, it is unlikely to do so in vivo. The mean diffusion distance in water for a small molecule in 2 s is approximately 60 μm: large compared with the likely distance between the phagosomal membrane and the captured bacterium, and vast compared with the internal span of the bacterium (~1 μm along its long axis). Therefore, nascent peroxynitrite has a lifetime easily sufficient to penetrate the captive cell and encounter a dehydratase target. Furthermore, if the cell contains 100 μM [4Fe-4S] clusters that react with peroxynitrite with the second-order rate constant that is exhibited in vitro by aconitase, then the half-time for the reaction between those clusters and peroxynitrite would be only 0.05 s. It would therefore appear that in E. coli peroxynitrite is much more likely to damage a dehydratase than to decompose.

The impact of the reactivity of HOONO* may be similarly diminished by the rate at which clusters scavenge ground-state peroxynitrite. Because the activation of peroxynitrite can be rate-limiting for HOONO*-dependent oxidations, less reactive sulfhydryls can largely preempt reactions between HOONO* and target molecules in vitro, and these clusters should do so in vivo as well. It is still possible, of course, that a minor HOONO* pathway will generate a lesion that has a disproportionate effect upon cell fitness. For example, HOONO* may directly damage DNA (64–66). Indeed, although low doses of peroxynitrite did not produce enough DNA damage to affect the viability of a wild-type cells, we observed that a strain defective in DNA repair was killed. However, the killing rate saturated near 100 μM peroxynitrite (Fig. 8), in parallel with the inactivation of dehydratases (Fig. 2). Because the extent of direct damage by HOONO* would presumably be first-order in dose, these data suggest that the DNA damage may have been a secondary effect of iron leakage from the damaged clusters. Attempts to test the involvement of iron in the DNA damage through the use of metal chelators were frustrated by the fact that the chelators directly scavenged the peroxynitrite. A possible scheme for metal-induced DNA damage in the absence of H2O2 might include the generation from peroxynitrite of an electrophilic nitronium cation (5, 13, 67). This mechanism has been...
This technique certainly offers the opportunity to determine whether from cluster destruction by superoxide or deregulation of the iron uptake system, result in an extreme sensitivity to DNA damage and possibly protein damage by both endogenous and exogenous oxidants. The polynsaturated lipids of eukaryotic membranes are vulnerable to iron-catalyzed peroxidation, and so mammalian cells may additionally be threatened with loss of membrane integrity as a secondary effect of iron spillage (69). Therefore it will be of interest to identify the mechanism that clears the iron. The clearance appeared to occur in two steps in these experiments: an extremely rapid phase that scavenged most of the detectable iron within 30 s, and a slower phase that removed the remainder over a period of minutes. A simple interpretation would be that the majority of the iron is initially either solvated or weakly bound to biomolecules from which the scavenging system can quickly remove it. A minor fraction of the iron may bind more strongly to charged biomolecules (possibly including the phosphodiester backbone of DNA) and only be re-captured by the scavenging system after it dissociates, which would be a slower process. The free-iron essay used in these experiments relies on desferrioxamine to chelate the iron. Because desferrioxamine is able to remove iron even from stronger ligands (including DNA), both iron populations would be detectable. We are hopeful that the EPR method can be used to test the involvement of Nif homologues in iron clearance.

**Physiological Significance of Cluster Damage by Peroxynitrite**—Peroxynitrite is generated by mammalian cells of several types, and its diffusion distance is sufficient that local tissue may be traumatized. At this date, only the mitochondrial aconitase is known to be acutely sensitive (26). The consequence of its inactivation, of course, would be the loss of oxidative phosphorylation, which would be metabolically catastrophic. Damage to mitochondrial DNA, membranes, and perhaps other proteins could occur consequent to its spillage of iron.

Equally intriguing is the role that peroxynitrite may be designed to play in the killing of phagocytosed cells. The oxidative burst of phagocytes damages the DNA of the captured cell (70). The stimulated phagosome generates both nitric oxide and superoxide, but it is improbable that these species damage DNA directly. However, the simultaneous production of these species in close quarters drives them to react together, forming peroxynitrite, while an additional fraction of superoxide dismutes to generate hydrogen peroxide (4–7). Both of these latter species cross membranes easily. The peroxynitrite can then attack the labile dehydratases. The immediate result of those injuries would be the dysfunctions that are also apparent in SOD-defective bacteria: a loss of tricarboxylic acid cycle activity (17), failure of branched-chain amino acid biosynthesis from the inactivation of dihydroxyacid dehydratase (20), and loss of the Entner-Doudoroff pathway from damage to 6-phosphogluconate dehydratase (18). These injuries are bacteriostatic but not in themselves lethal. However, the leaching of iron from these dehydratases could sensitize the cell to oxidative DNA damage at the very time that it is inundated with hydrogen peroxide. This concerted one-two punch may comprise a scheme to attack DNA, the weak link in the survival of the organism. In this context, it seems appropriate that the bacterium assigns a priority to iron clearance, completing this task far more rapidly, for example, than it repairs the damaged enzymes. These speculations will become testable when the genes associated with iron scavenging are identified.

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