Free iron in bacteria

by

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Photo: Filamentation of *E. coli* after oxidative DNA damage

The problem with intracellular "free" iron

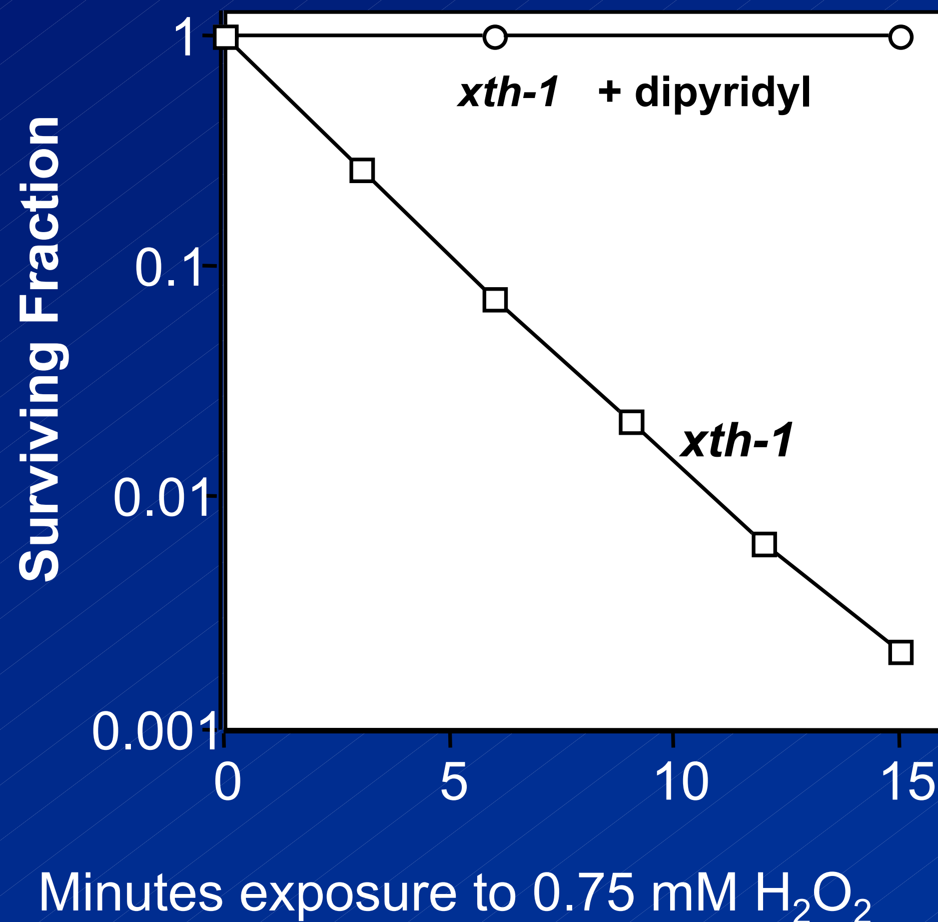
Most biological molecules cannot be damaged at a significant rate by direct reactions with molecular oxygen, superoxide, or hydrogen peroxide. However, they can be oxidized by hydroxyl radical ($HO^\bullet$). This species is formed when a single electron is transferred to H_2O_2

In *in vitro* systems the most facile donors of single electrons to H_2O_2 are transition metals, most notably iron (II) and copper (I).

Although organic electron donors, such as reduced quinones, are not thermodynamically prohibited from transferring electrons to H_2O_2, they are kinetically limited. No examples of such "organic Fenton reactions" have yet withstood scrutiny (but see [2]). Therefore, the vulnerability of intracellular DNA and proteins to oxidation should depend in part upon the concentration of available iron and copper.

Iron catalyzes HO• formation in vivo (1)

- Although either copper or iron suffices for H_2O_2 reduction *in vitro*, iron is the responsible species *in vivo*.
- There are three primary pieces of evidence that support this conclusion:
  a) First, iron chelators that can penetrate bacteria--dipyridyl, o-phenanthroline, and desferrioxamine--prevent exogenous H_2O_2 from damaging DNA [3]. In this figure dipyridyl fully prevents H_2O_2 from killing a strain of *E. coli* that cannot repair oxidative DNA lesions. The same result is obtained from direct measurements of DNA lesions.



Iron catalyzes $HO^{\bullet}$ formation in vivo (2)

b) Second, the kinetics with which H_2O_2 damages intracellular DNA indicate the mediation of a ferryl radical (FeO^{2+}). Ferryl radicals are the immediate products of electron transfer from Fe^{2+} to H_2O_2 [4]; they subsequently dissociate to form $Fe^{3+} + HO^{\bullet}$:

$$Fe^{2+} + H_2O_2 \text{ ----> } [FeO^{2+}] + OH^- + H^+ \text{ ----> } Fe^{3+} + HO^{\bullet}$$

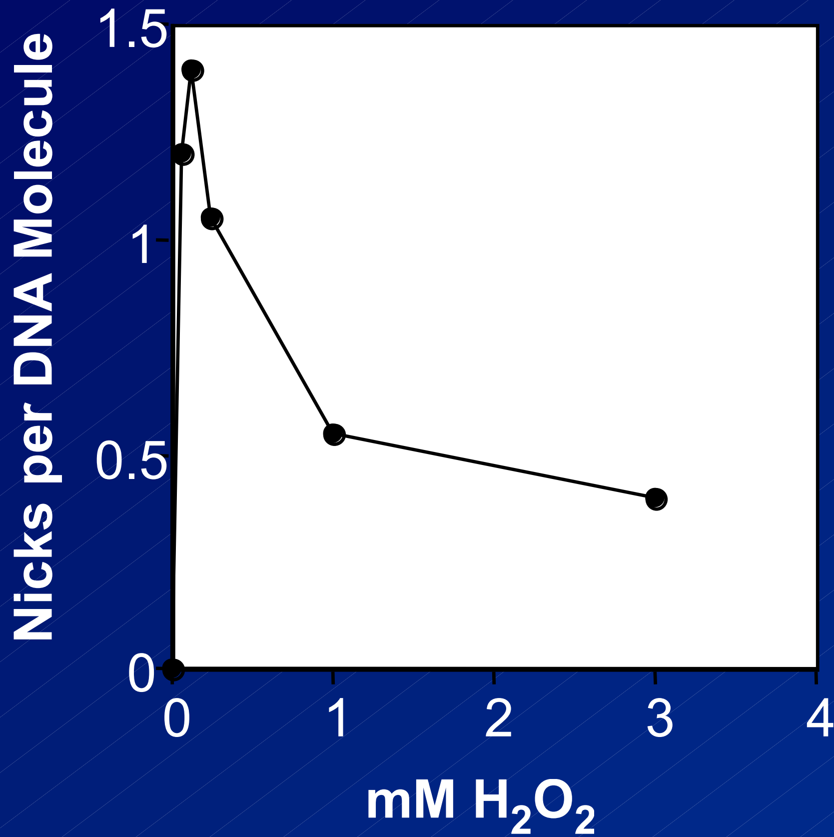
High concentrations of H_2O_2 can scavenge ferryl radicals before $HO^{\bullet}$ is formed:

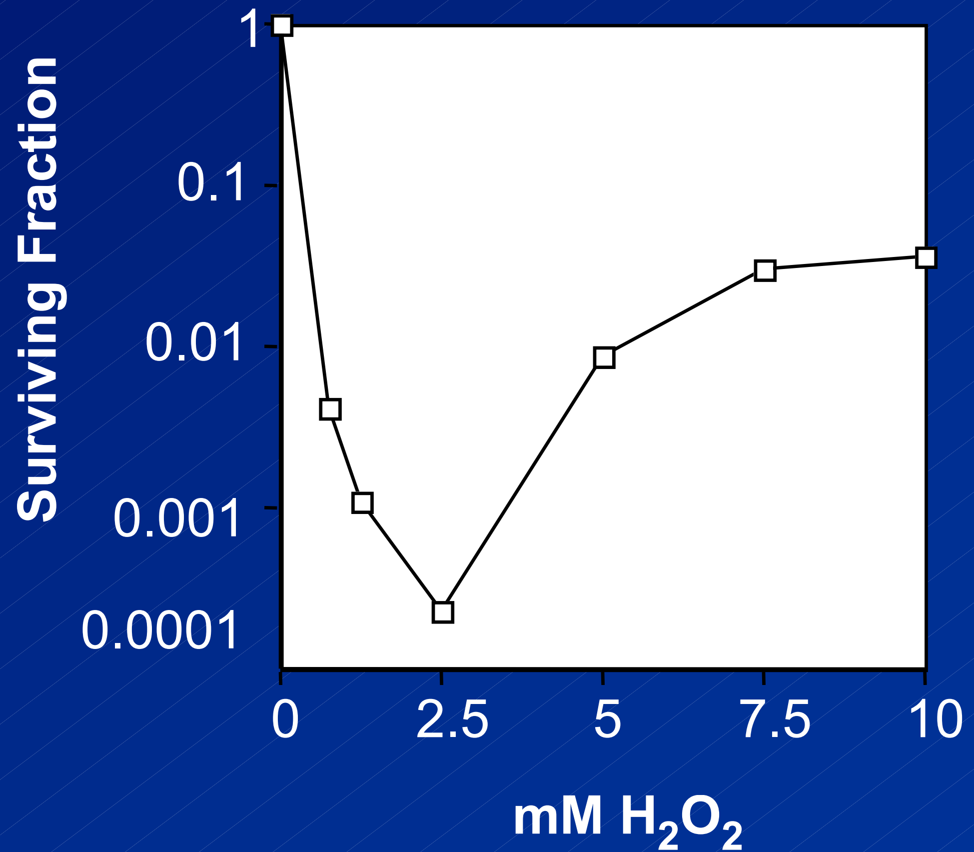
$$FeO^{2+} + H_2O_2 \text{ ----> } Fe^{3+} + H_2O + O_2^{\bullet -}$$

DNA damage is therefore actually more abundant at lower concentrations of H_2O_2 (left graph, next slide). This is not true of the copper-mediated reaction (not shown).

When intact cells are exposed to H_2O_2 , high concentrations of H_2O_2 again suppress the rate of DNA damage (right graph, next slide), thus indicating that $HO^{\bullet}$ formation inside cells is mediated by iron rather than copper [5].

[2, cont'd] **Damage suppression by excess H_2O_2 indicates the mediation of FeO^{2+}**





Iron catalyzes $HO^{\bullet}$ formation *in vivo* (3)

c) Third, *E. coli* mutants that over-import iron are unusually vulnerable to DNA damage by exogenous H_2O_2 [6, 7]. Overexpression of ferritin, a storage protein that specifically sequesters iron, prevents damage [6].

Why doesn't copper contribute to $HO^{\bullet}$ formation *in vivo*? The amount of available copper may be too small. However, even mutants that have lost the ability to control copper levels exhibit normal resistance to H_2O_2 [8]. Thus, a second factor may be that copper is liganded by the large pool of intracellular thiols, including glutathione. Millimolar levels of glutathione block the participation of copper in $HO^{\bullet}$ formation *in vitro* [9].

“Free” iron is the source of toxic hydroxyl radicals

Most iron inside cells is stably incorporated into proteins. Some of this iron is solvent-exposed and can be oxidized by H_2O_2. This is true, for example, of dehydratases that contain surface-exposed iron-sulfur clusters [10]. It follows that the $HO^{\bullet}$ that is formed by this reaction could potentially oxidize the side chains of these iron-binding proteins. However, the immense reactivity of $HO^{\bullet}$ precludes the possibility that it will diffuse far from the site of its formation before it reacts with a biomolecule or metabolite. Thus, protein-integrated iron is unlikely to generate the $HO^{\bullet}$ that damages DNA. These considerations suggest that the iron that catalyzes DNA damage is likely to be adventitiously localized on the surface of DNA or bound to small metabolites that can diffuse close to the DNA [11]. This iron is commonly denoted "free iron," to indicate that it is not integrated into enzymes.

- The term "free iron" is not intended to suggest that the iron is hexa-aqueous. Iron binds avidly to virtually all biomolecules, so iron atoms free within the cell are likely to adhere to the surfaces of membranes, nucleic acids, proteins, *etc*.

Intracellular free iron can be quantified

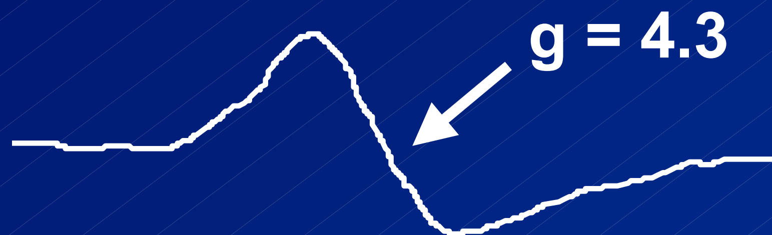
- Total metal analyses can quantify the amount of iron inside cells, but most of that iron is stably incorporated into proteins and is uninvolved in Fenton chemistry. To focus specifically upon free iron, either Mossbauer [12] or epr [13] methods are preferable. An important advantage of these methods is that they can be applied to whole cells.

- EPR is the more convenient of the two techniques. This method most easily detects ferric iron. (Ferrous iron is also epr-active but it displays a broad, indistinct signal.) Recent studies indicate that most of the free iron in *E. coli* is in the reduced form and therefore relatively invisible to epr [14]. However, the iron can be oxidized by treatment of the cells with either H_2O_2 or desferrioxamine. The latter agent binds and lowers the reduction potential of iron, triggering its autoxidation and trapping it in the ferric state. Thus exposure of *E. coli* to either of these agents allows the free iron pool to be visualized.

Free iron levels in E. coli

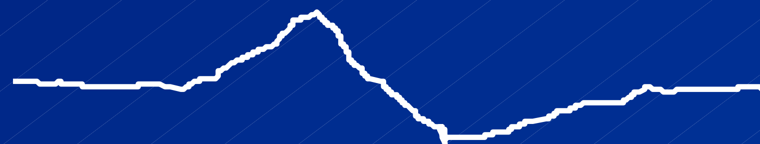
- Such experiments indicate that growing *E. coli* cells contain 15-30 micromolar free iron [13]. In such an experiment, the functional definition of "free iron" is iron that is redox-active and that it can be chelated by desferrioxamine. Since desferrioxamine blocks DNA damage, this includes the iron the catalyzes DNA damage.

- The Fur mutants that have unregulated iron uptake, and that are highly vulnerable to DNA damage, contain approximately 90 micromolar free iron [13].



Ferric iron standard

Wild-type cells: ca. 20 uM iron



Fur mutant: 85 uM iron

EPR spectra of Fe(III)desferrioxamine, *i.e.* ferrioximine. With a typical x-band epr these signals are centered at about 1550 gauss; $\Delta Hpp \approx 50$ G.

Why is there free iron in the cell?

- In no organism do we understand the route by which iron is trafficked from transport-complexes to its ultimate destination in metalloproteins. However, it seems inconceivable that iron is merely dumped into the cell upon import -- not only because free iron catalyzes oxidative damage, but also because iron sticks so avidly to biomolecules that it might never find its way to the target proteins. This is an important gap in our understanding of iron metabolism.

- This argument implies the existence of a pipeline of iron flow from transporter to incorporation. If this is correct, then free iron represents iron that has escaped the usual pathways of iron traffic.

Oxidants release iron from some [4Fe-4S] clusters

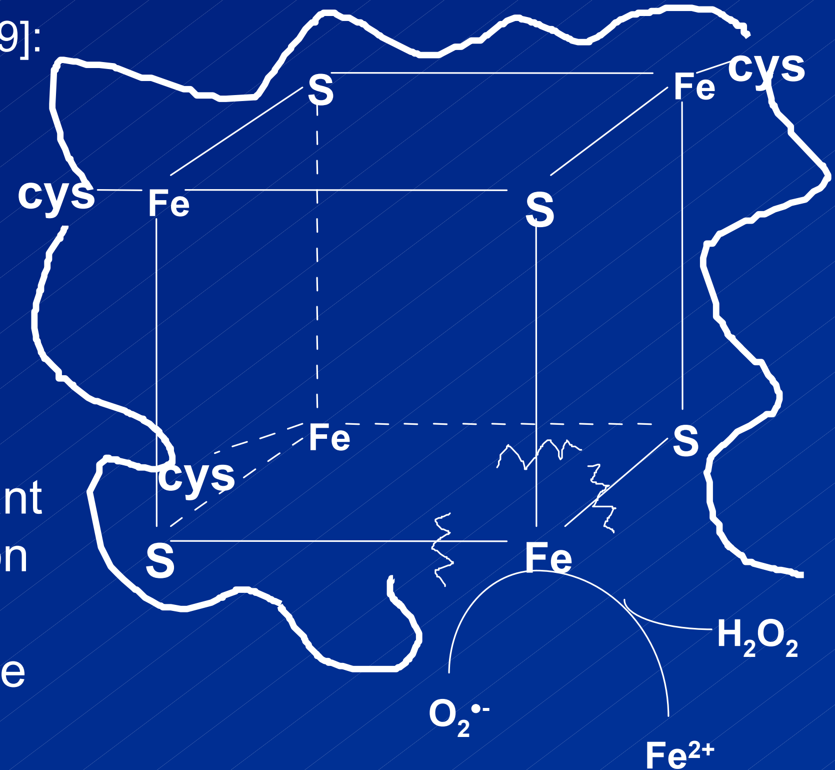
- One mechanism of escape is through disintegration of protein iron-sulfur clusters. In particular, clusters in dehydratases fall apart when they are oxidized into an unstable valence [15-19]:

$$\text{Protein-[4Fe-4S]}^{2+} + O_2^{\bullet -} + 2H^+ \text{ ---->}$$

$$\text{Protein-[4Fe-4S]}^{3+} + H_2O_2$$

$$\text{Protein-[4Fe-4S]}^{3+} \text{ --->}$$

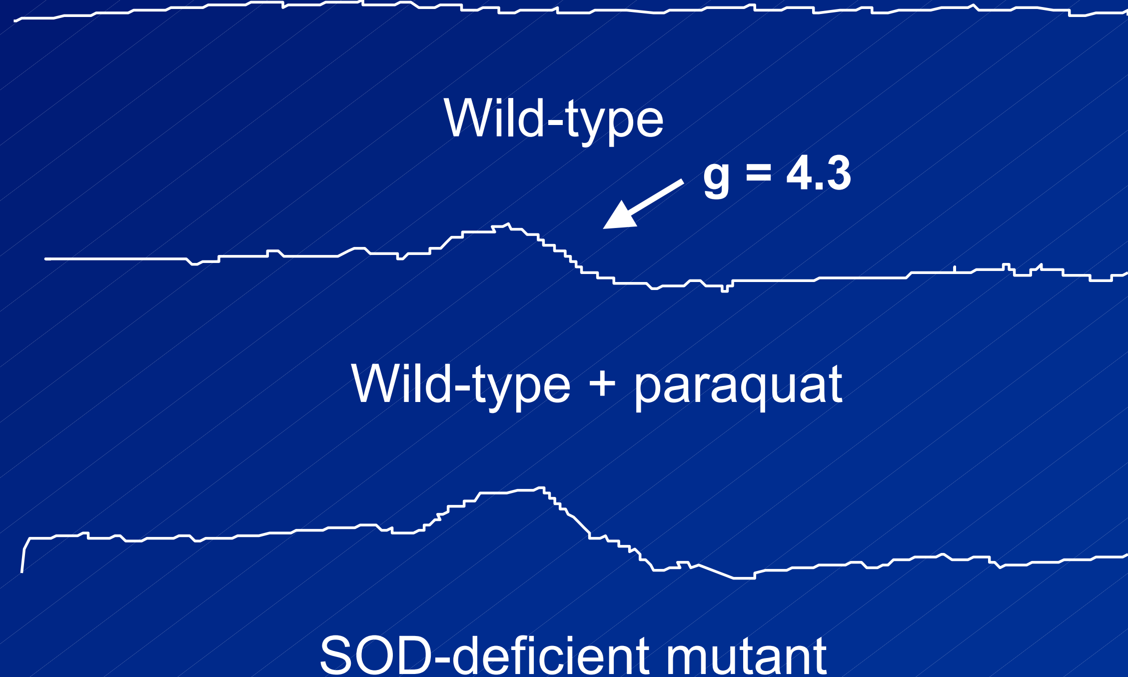
$$\text{Protein-[3Fe-4S]}^{1+} + Fe^{2+}$$



- Superoxide is a particularly good oxidant (right); the rate constant for this reaction is ca. 10^6 m^{-1} s^{-1}[10]. Peroxynitrite also rapidly destabilizes the clusters of these enzymes [20, 21]. H_2O_2 itself does so, but more sluggishly [10].

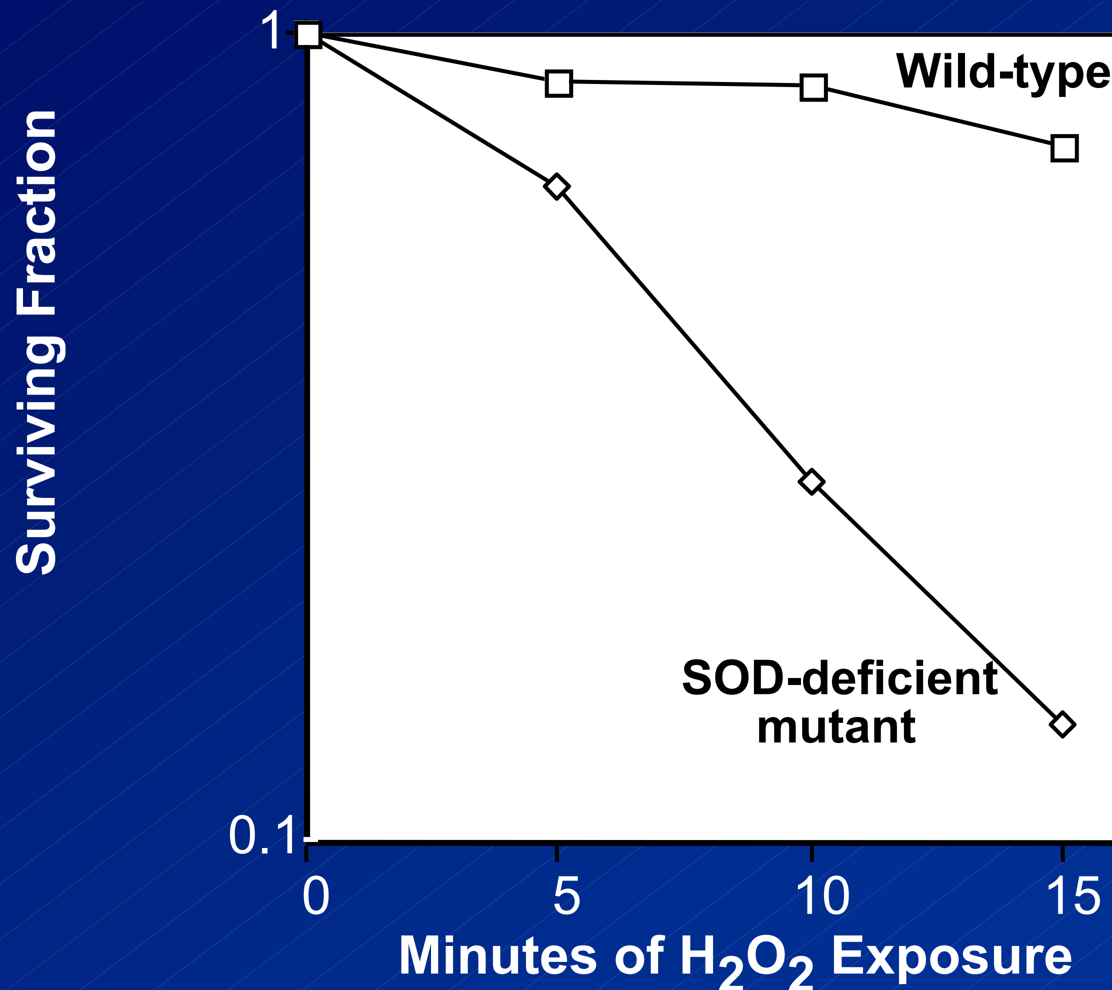
Free iron during oxidative stress

- The level of free iron is elevated when *E. coli* is exposed to redox-cycling drugs that generate superoxide. Mutants that lack SOD contain almost 10 times the amount of free iron as do wild-type cells [13]. For this reason, $HO^{\bullet}$ is formed at a proportionately higher rate when these cells are exposed to H_2O_2 [22]. Rapid DNA damage ensues (next slide).



(EPR spectra of ferrioximine are shown at equivalent gain.)

The abundant free iron in superoxide-stressed cells increases their vulnerability to DNA damage



Free iron without oxidative stress

- However, the free iron found in wild-type (SOD-proficient) cells does not arise from superoxide-mediated damage, since SOD overproduction cannot further diminish either the epr-detectable free-iron signal or the rate of $HO^{\bullet}$ formation. In fact, the amount of free iron is actually higher in anaerobic than aerobic *E. coli*, as the Feo iron-transport system is induced [7].

- This basal free iron may arise from spontaneous iron leakage from dehydratases or other proteins. Alternatively, iron may be trafficked by a weak chelator that does not preclude either its detection by epr or its participation in Fenton chemistry. It is notable that both Fur and aconitase B, two proteins that control iron acquisition, appear to bind iron reversibly, as if their regulatory action depends on the equilibration of iron between them AND an accessible iron pool in the cell [23, 24]. The nature of that pool remains unknown.

How does the cell control the amount of free iron?

- Given the role of free iron in creating DNA damage, it is unsurprising that bacteria have evolved methods to scavenge it. Experiments in which cells were exposed to a bolus of peroxynitrite revealed that free-iron levels rose and then fell within a minute [25]. The disappearance of the free iron exceeded the pace at which the damaged iron-sulfur clusters were repaired, suggesting that the free iron was scavenged.

Is Dps an iron scavenger?

- *E. coli* synthesizes three proteins--ferritin [26], bacterioferritin [27], and Dps [28] ; each sequester many atoms of iron. Ferritin and bacterioferritin are synthesized when iron is highly available in the environment, and thus they appear to be the routine storehouses of iron. They presumably donate the stored iron to metallation processes when iron becomes scarce. Consistent with this idea, mutants that lack these proteins cease growth more rapidly than wild-type cells when iron-starvation is imposed [29].

- In contrast, Dps is induced by the OxyR regulatory protein specifically in response to the presence of H_2O_2 [30]. Mutants that lack Dps are particularly sensitive to oxidative DNA damage [31]. *In vitro* this protein can both store iron and bind to DNA. Its protective role *in vivo* may be stem from a combination of these activities.

Outlook

- **The chemistry of oxidative damage:** iron leakage from oxidized dehydratases and its participation in Fenton chemistry is likely to be the same in all organisms. These processes, for example, have also been observed in yeast and in mammalian cells [32-35]. It is notable, though, that several bacteria have few (or no) iron enzymes and therefore may be exempt from this kind of damage [36, 27]. The vulnerability of still other organisms to H_2O_2 varies widely [38], perhaps reflecting differences in their free-iron content.

- **Future work:** despite the sophisticated biochemical and genetic strategies that can be brought to bear upon bacteria, we still know remarkably little about the physical mechanisms of iron transport, storage, and regulation, and virtually nothing about iron trafficking and its insertion into metalloproteins. These areas are ripe for future work.

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