Yeast Lacking Superoxide Dismutase(s) Show Elevated Levels of “Free Iron” as Measured by Whole Cell Electron Paramagnetic Resonance*

Received for publication, May 17, 2000, and in revised form, June 30, 2000
Published, JBC Papers in Press, July 5, 2000, DOI 10.1074/jbc.M004239200

Chandra Srinivasan‡, Amir Liba‡, James A. Imlay§, Joan Selverstone Valentine‡, and Edith Butler Gralla¶¶

From the ‡Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90095-1569 and the §Department of Microbiology, University of Illinois, Urbana, Illinois 61801

A current hypothesis explaining the toxicity of superoxide anion in vivo is that it oxidizes exposed [4Fe-4S] clusters in certain vulnerable enzymes causing release of iron and enzyme inactivation. The resulting increased levels of “free iron” catalyze deleterious oxidative reactions in the cell. In this study, we used low temperature Fe(III) electron paramagnetic resonance (EPR) spectroscopy to monitor iron status in whole cells of the unicellular eukaryote, Saccharomyces cerevisiae. The experimental protocol involved treatment of the cells with desferrioxamine, a cell-permeant, Fe(III)-specific chelator, to promote oxidation of all of the “free iron” to the Fe(III) state wherein it is EPR-detectable. Using this method, a small amount of EPR-detectable iron was detected in the wild-type strain, whereas significantly elevated levels were found in strains lacking CuZn-superoxide dismutase (CuZn-SOD) (sod1Δ), Mn-SOD (sod2Δ), or both SODs, throughout their growth but particularly in stationary phase. The accumulation was suppressed by expression of wild-type human CuZn-SOD (in the sod1Δ mutant), by pnm1, a genetic suppressor of the sod1 mutant phenotype (in the sod1Δsod2Δ double knockout strain), and by anaerobic growth. In wild-type cells, an increase in the EPR-detectable iron pool could be induced by treatment with paraquat, a redox-cycling drug that generates superoxide. Cells that were not pretreated with desferrioxamine had Fe(III) EPR signals that were equally as strong as those from treated cells, indicating that “free iron” accumulated in the ferric form in our strains in vivo. Our results indicate a relationship between superoxide stress and iron handling and support the above hypothesis for superoxide-related oxidative damage.

Superoxide dismutases are antioxidant enzymes that disproportionate superoxide (O2·−) (1) to hydrogen peroxide (H2O2) and dioxygen (2–5). This class of enzyme is found in almost all aerobic organisms (6). Eukaryotes, including Saccharomyces cerevisiae, contain Mn-SOD (product of the SOD2 gene) in the matrix of the mitochondria, and CuZn-SOD (product of the SOD1 gene) elsewhere in the cell, most notably in the cytoplasm, nucleus and lysosomes. Yeast cells lacking either SOD gene are viable but compromised in various ways. Yeast sod1Δ mutant strains grow poorly in air, are very sensitive to redox-cycling drugs such as paraquat or menadione, die quickly in stationary phase, and exhibit lysine and methionine auxotrophies when grown aerobically. sod2Δ mutants are oxygen-sensitive and, when required to respire, grow poorly and are particularly sensitive to paraquat. The double sod1Δsod2Δ mutant is more severely compromised, exhibiting essentially a summation of the single mutant phenotypes (7–9).

We have been searching for explanations for the severity of the sod1 mutant phenotype in yeast, and thus for the toxicity of superoxide in vivo. Superoxide is more selective in its chemical reactions than most other reactive oxygen species and thus less likely to exert its toxicity through generalized oxidative damage reactions of the type characteristic of hydroxyl radicals, for example. Recently a particularly attractive mechanism of superoxide toxicity was proposed (10), based on the observation that superoxide can very specifically oxidize exposed [4Fe-4S] clusters in certain enzymes, causing release of iron from the cluster and inactivation of the enzyme (3, 11–18). This process leads to further oxidative damage of other cellular components, as “free iron” can promote, via the Fenton reaction, the formation of hydroxyl radical (‘OH) (2, 10, 19–21). Thus, in this scenario, superoxide is involved in the Fenton reaction by providing the necessary iron catalyst, and SOD both protects the [4Fe-4S] enzymes from inactivation and prevents the accumulation of excess intracellular iron. Support for this hypothesis has come from two directions.

First, there is a demonstrated class of enzymes that contain exposed, superoxide-sensitive [4Fe-4S] cluster, the best-studied example of which is mitochondrial aconitase, which catalyzes the conversion of citrate to isocitrate in the citric acid cycle. Other such enzymes include homoaconitase (involved in lysine biosynthesis), isopropyl malate isomerase (Leu1p) (in the leucine biosynthetic pathway), and IRP1 (involved in iron sensing and response in many eukaryotes, but not yeast) (22–24). The sensitivity of aconitase activity to superoxide has been exploited as a method to measure steady state levels of superoxide in vivo (25, 26).

Second, altered iron status has been demonstrated in organisms lacking SOD (21, 27, 28). Escherichia coli cells lacking SOD have been shown to have elevated levels of iron that can...
be chelated in vivo by the cell-permeant chelator desferrioxamine and detected by whole cell Fe(III) EPR (21). This pool of chelatable iron or “free iron” is clearly distinguishable from the bulk of cellular iron that is tightly bound to proteins and not accessible to the chelator. In untreated E. coli, the “free iron” is present as Fe(II) (EPR silent); upon addition of desferrioxamine the Fe(II) is bound and converted to Fe(III), which is EPR-detectable. This pool of Fe(II) was shown to accelerate DNA damage (21). In addition, we have found evidence that a similar process may occur in eukaryotes: yeast lacking SOD have an increased requirement for iron in aerobic growth and show increased iron uptake and accumulation under certain growth conditions (28).

In the present study, we have extended this work to eukaryotes and adapted the in vivo whole cell Fe(III) EPR methodology to examine the iron status of yeast lacking either or both of the SODs. Our data show that superoxide stressed yeast (either sodΔ mutants or paraquat-treated wild-type) have elevated levels of EPR-detectable iron, which is present in the Fe(III) state.

**EXPERIMENTAL PROCEDURES**

**Reagents, Media, and Cell Growth**—The yeast strains used in this study are described in Table I (29–31). Desferrioxime mesylate (commonly known as desferrioxamine) and methyl viologen (paraquat) were purchased from Sigma. Yeast were cultured either in rich medium (YP; typically, duplicate 2–3-ml samples from 50-ml stationary phase cultures were collected and washed once with 10 ml of cold 20 mM Tris-Cl, pH 7.4. The cells were incubated at 30 °C with shaking for 15 min and then centrifuged, 0.5 or 1.0 ml was diluted to 7 ml in metal-free water and subjected to analysis using a Thermo-Jarrell Ash Iris 1000 inductively coupled plasma atomic emission spectrometer.

**EPR Sample Preparation**—The procedure for EPR sample preparation was adapted from Keyer and Imlay (21). After 72 h of growth, the final Fe(III) standard along with the intracellular yeast cell volume of 70 μm3 (33) and the number of cells used for EPR sample preparation were used to quantitate the iron levels inside yeast cells. Reported numbers are averages of at least two independent samples.

**Measurement of Total Iron**—Cells were grown as described above. Typically, duplicate 2–3-ml samples from 50-ml stationary phase cultures were collected by centrifugation (5 min at 4000 rpm) and washed once with 10 ml of 10 mM EDTA, pH 8, and twice in 5 ml of metal-free water. The A600 and exact volume of the cell suspension on the last wash were recorded. The final cell pellet was then resuspended in 1 ml of 10% ultrapure nitric acid and heated at 98 °C for 18 h. After complete digestion, 0.5 or 1.0 ml was diluted to 7 ml in metal-free water and the Fe(III) standard was measured on the same day, revealed that the yeast wild-type strain consistently has a very weak Fe(III) EPR signal at g = 4.3. Expression of human CuZn-SOD or the CuZn-SOD standard formula

| Strain                | Genotype                        | Source |
|-----------------------|---------------------------------|--------|
| EG103 (wild-type)     | MATa leu2 his3 trp1 ura3         | Ref. 29|
| EG110 (sod2Δ)         | MATa leu2 his3 trp1 ura3 sod2Δ:TRP1 | Ref. 29|
| EG118 (sod1Δ)         | MATa leu2 his3 trp1 sod1Δ:URA3   | Ref. 29|
| EG133 (sod1Δ sod2Δ)   | MATα leu2 his3 trp1 sod1Δ:URA3 sod2Δ:TRP1 | Ref. 29|
| VCSUP1 (sod1Δ sod2Δ pmr1) | Same as EG133 plus sod1Δ–I     | Ref. 30|
| 118-hWT               | EG118 with plasmid YEp551-hSOD1 (expressing human CuZn-SOD) | Ref. 31|

**RESULTS**

**Yeast Lacking CuZn-SOD Have Elevated Levels of EPR-detectable Iron, and Normal Levels Are Restored by Expression of Human CuZn-SOD**—Wild-type yeast and the sod1Δ mutant were grown to stationary phase (72 h) in SDC and treated (15 min) with desferrioxamine to convert “free iron” to the Fe(III) state. Whole cell low temperature Fe(III) EPR spectra were obtained and are shown in Fig. 1A. A large signal at g = 4.3 was observed for the sod1Δ strain, but the presence of either yeast or human CuZn-SOD was enough to largely prevent its appearance. Quantitation by double integration, comparing to spectra of iron standards measured on the same day, revealed that the yeast wild-type strain had a basal level of EPR-detectable iron of 12.8 ± 1.4 μM, whereas the isogenic sod1Δ strain had a level that was 5-fold higher (49.2 ± 0.15 μM). Expression of the human CuZn-SOD in the sod1Δ mutant, which is known to fully restore that strain to a wild-type phenotype, also prevented the elevation of EPR-detectable iron (level was 15.7 ± 2.6 μM).

The Fe(III) EPR signal at g = 4.3 is characteristic of ferric iron in a high spin complex (see Fig. 1 for examples of spectra). Most protein-bound iron does not give rise to an EPR signal at this g value (34), and as has been shown in E. coli, desferrioxamine treatment does not remove iron from proteins (21). Evidence that this is also the case in S. cerevisiae comes from the fact that our wild-type strain consistently has a very weak Fe(III) EPR signal when compared with the CuZn-SOD (sod1Δ) knockout strain, although they both contain normal amounts of iron.

The EPR signal was not affected by variation in the level of iron in the medium used for desferrioxamine incubation. We tested three different incubation media—YP medium, rich in iron; SD medium, low in iron; and 20 mM Tris-Cl, pH 7.4, buffer, very low in iron—for the 15-min incubation of cells with desferrioxamine and saw no significant difference in the Fe(III)
EPR signal intensity with any of these media (data not shown).

To rule out the possibility that this effect was specific to one culture medium or one growth stage, Fe(III) EPR measurements were also conducted on samples prepared from log phase cultures (A550 = 0.3–0.5, at least 5 doublings short of the maximum density) grown in either YPD or YPG. In log phase, cells lacking SOD had smaller increases, around 2-fold, in EPR-detectable iron. Due to the requirement for large culture volumes (1–2 liters for log phase versus 10–15 ml for stationary phase experiments) and the smaller difference in the EPR-detectable iron levels between the wild-type and the mutants, the log phase growth conditions were not pursued further. Results similar to those with the 72-h samples were obtained when cells were grown for 24 h (data not shown), but the most consistent effects were seen in 72-h cultures, so we continued with those conditions.

A Genetic Suppressor of the sod1Δ sod2Δ Phenotype Lowers the Level of EPR-detectable Iron—Several second site suppressors of the defects of the yeast sodΔ mutants have been identified (29, 30, 35–37). Of these, the most common is pmr1. Mutations in PMR1, a Golgi P-type ATPase that plays a role in calcium metabolism, have been shown to rescue S. cerevisiae that lack SOD activity, probably by causing the accumulation of manganese, which can scavenge superoxide (35). If elevated EPR-detectable iron is causally related to the phenotype of sod1Δ sod2Δ mutants, we reasoned that this suppressor would restore iron levels to normal.

We tested the effect of the pmr1 mutation on iron accumulation in the yeast sod1Δ sod2Δ double knockout strain. Fig. 1B shows the EPR spectra for wild-type, sod1Δ sod2Δ, and sod1Δ sod2Δ pmr1 yeast strains grown for 72 h and treated with desferrioxamine. The EPR-detectable iron level in the sod1Δ sod2Δ strain was more than 7-fold higher than that of the wild-type. For duplicate samples done on the same day, the level for wild-type was 10.5 ± 0.6 μM, and for the sod1Δ sod2Δ mutant it was 64.5 ± 13.8 μM. The presence of the pmr1 mutation in the sod1Δ sod2Δ strain decreased the EPR-detectable iron level to 26.9 ± 3.0 μM, much lower than in the sod1Δ sod2Δ strain, but still approximately 3-fold higher than the level seen in the wild-type strain. This result indicates that the pmr1 mutation can partially suppress the elevated EPR-detectable iron phenotype observed in sod1Δ sod2Δ strain, which correlates neatly with the fact that the pmr1 mutation does not completely suppress the other phenotypes of the sod1Δ sod2Δ strain.

EPR-detectable Iron and Total Iron Are Elevated in All Yeast Strains That Lack SOD(s)—Absence of either CuZn-SOD or Mn-SOD caused significant increases in EPR-detectable iron. Fig. 2 shows a summary of all our data for the sod1Δ mutants grown for 72 h. The sod1Δ mutant typically had around 5-fold elevation in EPR-detectable iron, the sod2Δ mutant had around 4-fold elevation, and the sod1Δ sod2Δ double knockout strain had around 9-fold elevation but also showed greater variability from experiment to experiment. Total iron also increased somewhat under these conditions (72-h growth period), as was previously reported for a shorter growth period of 18 h (28), and this increase in total iron can be fully explained by the increase in “free iron.” In the wild-type and single mutants, the “free iron” pool constitutes a relatively small fraction of the total iron—8% for wild-type and 20–25% for the single mutants—and the increased deposition of iron into the EPR-detectable pool in the single mutants is adequately compensated for by the increase in total iron (i.e. non-EPR-detectable iron is similar in wild-type and the single mutants). On the other hand, in the double mutant, the EPR-detectable pool reaches nearly 50% of the total iron, and the cells are apparently unable to fully compensate, so that the amount of non-EPR-detectable iron is lower in the sod1Δ sod2Δ mutant than it is in wild-type.

Superoxide-stressed Wild-type Cells Have Elevated EPR-detectable Iron—If the increase in EPR-detectable iron in yeast lacking SOD is connected with elevated O2− production, then it should be possible to replicate the effect in wild-type cells by increasing their O2− production. Therefore, we treated wild-type cells with paraquat and measured EPR-detectable iron. Para-
Fig. 3. Effect of treatments that alter superoxide levels on EPR-detectable iron levels. A, paraquat treatment was used to elevate superoxide concentration. Wild-type cells were grown in air in SDC medium with or without 10 mM paraquat for 24 h. Values reported are averages from three independent cultures. B, anaerobic growth was used to decrease superoxide production. Parallel cultures of wild-type and sod1Δ sod2Δ mutants were grown in air and under anaerobic conditions for 24 h. EPR sample preparation was performed under aerobic conditions as before. Average EPR-detectable iron values from two independent cultures are reported.

Paraquat is a redox-cycling drug that generates superoxide in vivo. Although very low levels of paraquat (10 μM) are toxic to sod1Δ mutant yeast, wild-type strains tolerate much higher concentrations (up to 50 mM under some conditions), although growth is slowed at very high paraquat concentrations. Wild-type cells cultured in medium containing 10 mM paraquat for 24 h had at least a 5-fold increase in EPR-detectable iron per cell compared with untreated cells (Fig. 3A). A significant increase in EPR-detectable iron was also seen with 1 mM paraquat, a concentration at which the growth rate was only slightly affected (data not shown). These data support the hypothesis that the excess EPR-detectable iron is due to the deleterious effects of superoxide radical.

EPR-detectable Iron Does Not Accumulate under Anaerobic Growth Conditions—An important feature of the phenotype of sodΔ yeast strains is that it appears only under aerobic conditions. In order to see whether the elevated EPR-detectable iron seen in sodΔ mutants is due to the effect of aerobic growth, wild-type and sod1Δ sod2Δ strains were cultured in air and also anaerobically (under nitrogen) for 24 h. Under aerobic growth conditions, the sod1Δ sod2Δ double mutant had about 6-fold higher EPR-detectable iron than did the wild-type, whereas under anaerobic conditions, its level of EPR-detectable iron was not significantly different from that of the wild-type (Fig. 3B). These data clearly indicate that the elevated EPR-detectable iron seen in sod1Δ sod2Δ mutant is dependent on the presence of oxygen.

The Excess “Free Iron” Is in the Fe(III) State in Yeast Lacking SOD—in the experiments described so far, we used desferrioxamine to chelate all loosely bound or “free iron” within the cells and convert any Fe(II) present to Fe(III) so that it would be detectable by Fe(III) EPR. (Unlike Fe(III) EPR signals, Fe(II) signals are very broad and difficult to detect.) In order to determine the proportion of iron present as Fe(II), we conducted Fe(III) EPR measurements on identical samples with and without desferrioxamine treatment. To our surprise, the EPR signals seen in untreated and desferrioxamine-treated samples of all the strains tested were practically identical (Fig. 4). Similar results were also obtained for the wild-type and the sod1Δ sod2Δ mutant when incubation was shortened to 24 h (data not shown). These data indicate that in yeast, unlike in E. coli, most, if not all, of the EPR-detectable iron is present in the Fe(III) state.

Fig. 4. Oxidation state of the EPR-detectable iron in vivo. Wild-type, sod1Δ, sod2Δ, and sod1Δ sod2Δ strains were cultured for 72 h in SDC medium and split into two equal parts. One part was treated with desferrioxamine (black bars), whereas the other was not (gray bars), and iron was measured by EPR as before. Data shown are averages of three independent measurements for each.

DISCUSSION

In this study, we explored the iron status of yeast lacking CuZn-SOD and/or Mn-SOD using an EPR method that measures loosely bound or “free iron,” as opposed to iron bound to protein; this same method had previously been applied to E. coli lacking SOD. This method is particularly advantageous for the study of in vivo status, as sample preparation is minimal and there is no need for cell lysis. This powerful, noninvasive method can be used to monitor the cellular oxidative status as well as to quantify a pool of labile iron.

Accumulation of EPR-detectable iron occurred under conditions that increase superoxide levels (in mutants lacking SOD(s)) and in wild-type yeast treated with paraquat and was not observed under conditions that minimize superoxide levels (anaerobic growth or expression of wild-type SOD). The facts that 1) longer culture periods increased the EPR-detectable iron level and 2) genetic changes—either overexpression of human Sod1p or the presence of a pmr1 mutation—decreased the EPR-detectable iron signal are good evidence that the Fe(III) EPR signals were due to intracellular, as opposed to extracellular, processes.

It is interesting to note that the single sod1Δ or sod2Δ mutant yeast are apparently able to compensate for loss of iron to the EPR-detectable pool by increasing their iron uptake (Ref. 28 and data in Fig. 2), so that the level of non-EPR-detectable (or “normal”) iron is similar to that of wild-type. The double sod1Δ sod2Δ mutant, on the other hand, is not able to fully compensate, losing nearly 50% of its iron to the EPR-detectable pool and showing a somewhat decreased absolute level of “normal” iron relative to wild-type. This observation may help explain the severity of the double mutant phenotype.

The original EPR method used for E. coli relied on a cell permeant chelator to convert all “free iron” to the Fe(III) form, which is detectable by EPR, giving a signal at g = 4.3. In E. coli, addition of the chelator was essential, indicating that in the cell, the iron was present as Fe(II) (21). A similar EPR method has been used to measure a “free iron” pool in rat tissues; this pool has also been identified as Fe(II) (38). In yeast, on the contrary, the “free iron” turned out to be detectable without adding chelator, indicating that in our experiments it is present in the Fe(III) state. We were surprised by this discovery, because of the previous results and because the intracellular environment is very reducing, leading one to expect that a pool of lost or misdirected iron would be in the Fe(II) state.

The EPR-detectable iron pool could originate from an abnormal process whereby iron released by superoxide stress is chelated by whatever metabolites are available (possibly citrate, which is relatively abundant and an excellent chelator) and then accumulates in an unusual pool. Alternatively, the signal
could originate from a normal iron storage complex that gives an EPR signal and the quantity of which is increased under superoxide stress conditions. Reasons for expansion of this pool could include a naturally slow rate of removal of iron from this pool or a superoxide-sensitive step in the reutilization pathway. By analogy with mammalian systems and from the fact that no iron export machinery has been described in yeast, it seems possible that there is a system to sequester “used” iron in an inactive form so that it does not catalyze further cell damage.

These data can thus be explained by a process of continuous, superoxide-driven loss of iron from [4Fe/4S] cluster enzymes necessitating resynthesis of the clusters, in which the iron released from the clusters accumulates in a form that is inaccessible to the biosynthetic machinery and iron must be newly imported for use in reconstituting the damaged clusters. Evidence for several aspects of this hypothesis has been accumulating. First, superoxide can efficiently oxidize certain enzymes with exposed [4Fe/4S] clusters, causing the release of iron and inactivation of the enzyme (18, 22). The activity loss has even been used to quantitate superoxide generation in several organisms (26). Second, superoxide stress increases iron demand in yeast (28) and in E. coli. In E. coli, iron lost from clusters is not normally used for resynthesis (39), and in both yeast and E. coli, sodΔ mutants are iron-deficient despite having higher levels of free iron (28, 40, 41).

Another aspect of this hypothesis is that the released iron is proposed to catalyze deleterious oxidative reactions (42). In E. coli, it has been demonstrated experimentally that iron released in such a process increases DNA damage (21). Very recently, it was shown that treatment of isolated mitochondrial aconitate with superoxide led to production of hydroxyl radical (43). We now demonstrate that an unusual pool of iron accumulates in sodΔ mutants of the eukaryote S. cerevisiae, which we postulate to be the iron released from the clusters. It appears likely that this mechanism contributes to the observed phenotypes; there may be other mechanisms as well.

In yeast, there are several known or potential Fe/S-containing enzymes that may “leak” iron in this way. Homoaconitate is inactivated in the sod1 strain, resulting in an air-dependent auxotrophy for lysine, and is located in the intermembrane space along with some of the CuZn-SOD.3 Leu1p is another Fe/S enzyme that is located in the cytoplasm (23) that could contribute as well. The classic superoxide-sensitive enzyme, mitochondrial aconitate, is present in yeast and is superoxide-sensitive: its activity is greatly reduced in an sod2Δ mutant strain after 72 h of growth (44). There are almost certainly other vulnerable enzymes as well. An EPR signal at g = 4.3 is characteristic of high spin, rhombic, mononuclear Fe(III). Examples of complexes of this type include iron citrate, transferrin, and oxidized rubredoxin, as well as the desferrioxamine complex (34). Because the desferrioxamine complex is not relevant here, questions arises as to what the signal-producing iron is complexed to in vivo. Most protein-bound iron does not give a signal at this g value; thus, we can eliminate heme iron, non-heme iron proteins, and Fe/S clusters from consideration. Nor does ferritin give much of a signal at this g value. In any case, no ferritin or transferrin has been reported to be present in yeast or identified in its genome sequence, so these possibilities are eliminated.

The subcellular location of the EPR-detectable iron is also a very important issue, and one that we are pursuing, but we have no definitive results at present. Iron metabolism in yeast is a very rapidly moving field at the moment, and recent results from other laboratories, hinting at either a cytoplasmic or vacuolar location, may be relevant to this question. The vacuole is known to be a storage site for many metabolites, including some metals. Evidence for vacuolar involvement in iron metabolism includes the following points. First, an iron transport system encoded by the FET5 and FTH1 genes has been found on the vacuolar membrane (45) and is homologous to the high affinity plasma membrane iron transport complex encoded by the FET3 and FTR1 genes (46). The authors suggest that iron is stored in ferric form in the vacuole and that the Fets/Fth1 complex might be involved in mobilizing intravacuolar stores of iron. Second, yeast lacking sod1 were shown to have a greater number of vacuoles (or to exhibit vacuolar fragmentation) in the presence of oxygen and excess iron (47). On the other hand, there is new evidence that under excess iron conditions, wild-type cells accumulate iron mostly in the cytosol, not in vacuole, prevacuole, or Golgi. The cytosolic iron is not sensed by the iron-responsive transcription factor Aft1p, which suggests that the iron is stored in an unreactive or inaccessible form (48).

Our data suggest that cellular iron status and superoxide levels are linked in yeast and that this organism may provide a useful model system for studies of disturbances in iron metabolism related to human diseases (49–54) and in vivo oxidative stress (24).

Acknowledgments—We express our sincere gratitude to Drs. Barney Bales and Miroslav Peric at California State University, Northridge, for the use of their EPR instrument and their willing and expert technical assistance. We also thank Dr. Alex Smirnov and Anh Nguyen for their help with EPR at the Illinois EPR center and Dr. Jim Roe for his valuable advice on interpreting spectra.

REFERENCES
1. Sawyer, D. T., and Valentine, J. S. (1981) Acc. Chem. Res. 14, 393–400
2. Fridovich, I. (1978) Science 201, 875–880
3. Fridovich, I. (1995) Annu. Rev. Biochem. 64, 97–112
4. Valentine, J. S., Wertz, D. L., Lyons, T. J., Liou, L.-L., Goto, J. J., and Gralla, E. B. (1998) Curr. Opin. Chem. Biol. 2, 253–262
5. Beyer, W., Imlay, J., and Fridovich, I. (1991) Proc. Nutric. Aces Res. Mol. Biol. 40, 221–253
6. McCord, J. M., Kelle, B. B., Jr., and Fridovich, I. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 1024–1027
7. Gralla, E. B., and Kosman, D. J. (1992) Adv. Genet. 30, 251–319
8. Gralla, E. B. (1997) in Cold Spring Harbor Monograph Series, No. 34: Oxidative Stress and the Molecular Biology of Antioxidant Defenses (Scandalios, J. G., ed.) pp. 495–525, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
9. Gralla, E. B., and Valentine, J. S. (1991) J. Bacteriol. 173, 5918–5920
10. Imlay, J. A., and Fridovich, I. (1994) Free Radic. Biol. Med. 16, 29–33
11. Gardner, P. R., and Fridovich, I. (1991) J. Biol. Chem. 266, 18328–18333
12. Gardner, P. R., and Fridovich, I. (1990) J. Biol. Chem. 265, 1478–1483
13. Gardner, P. R., and Fridovich, I. (1992) J. Biol. Chem. 267, 8757–8763
14. Gardner, P. R., and Fridovich, I. (1993) Arch. Biochem. Biophys. 301, 98–102
15. Imlay, J. A., and Fridovich, I. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5892–5896
16. Imlay, J. A., and Fridovich, I. (1993) Arch. Biochem. Biophys. 310, 379–384
17. Murakami, K., and Yoshino, M. (1997) Biochem. Mol. Biol. Int. 41, 481–486
18. Flint, D. H., Smyk-Randall, E., Tuminello, J. F., Draczynska-Lusiak, B., and Allen, R. M. (1996) J. Biol. Chem. 271, 29–33
19. Feng, L.-L., Liou, V. D., Longo, J. S., Valentine, J. S., and E. B. Gralla, manuscript in preparation.

3 L.-L. Liou, V. D. Longo, J. S. Valentine, and E. B. Gralla, manuscript in preparation.
33. Guthrie, C., and Fink, G. R. (1991) Methods Enzymol. 184
34. Benzini, A., and Gatteschi, D. (1999) in Inorganic Electronic Structure and Spectroscopy (Solomon, E. I., and Lever, A. B. P., eds) Vol. I, pp. 93–160, John Wiley & Sons, Inc., New York
35. Lapinskas, P. J., Cunningham, K. W., Liu, X. F., Fink, G. R., and Culotta, V. C. (1995) Mol. Cell. Biol. 15, 1382–1388
36. Lin, S. J., and Culotta, V. C. (1996) Mol. Cell. Biol. 16, 6303–6312
37. Strain, J., Lorenz, C. R., Bode, J., Garland, S., Smolen, G. A., Ta, D. T., Vickery, L. E., and Culotta, V. C. (1998) J. Biol. Chem. 273, 31138–31144
38. Kozlov, A. V., Bini, A., Gallesi, D., Giovannini, F., Iannone, A., Masini, A., Meletti, E., and Tomasi, A. (1996) Biometals 9, 98–103
39. Keyer, K., and Imlay, J. A. (1997) J. Biol. Chem. 272, 27652–27659
40. Benov, L., and Fridovich, I. (1998) J. Biol. Chem. 273, 10313–10316
41. Maringanti, S., and Imlay, J. A. (1999) J. Bacteriol. 181, 3792–3802
42. Lochev, S. L. (1996) Free Radic. Biol. Med. 25, 369–384
43. Vásquez-Vivar, J., Kalyanaraman, B., and Kennedy, M. C. (2000) J. Biol. Chem. 275, 14064–14069
44. Longo, V. D., Liou, L.-L., Valentine, J. S., and Gralla, E. B. (1999) Arch. Biochem. Biophys. 365, 131–142
45. Urbanowski, J. L., and Piper, R. C. (1999) J. Biol. Chem. 274, 38061–38070
46. Askwith, C., and Kaplan, J. (1999) Trends Biochem. Sci. 23, 135–138
47. Corson, L. B., Folmer, J., Strain, J. J., Culotta, V. C., and Cleveland, D. W. (1999) J. Biol. Chem. 274, 27590–27596
48. Chen, O. S., and Kaplan, J. (2000) J. Biol. Chem. 275, 7626–7632
49. Gerlach, M., Ben-Shachar, D., Riederer, P., and Youdim, M. B. H. (1994) J. Neurochem. 63, 793–807
50. Babcock, M., de Silva, D., Oks, R., Davis-Kaplan, S., Jiralerspong, S., Montermini, L., Pandolfi, M., and Kaplan, J. (1997) Science 276, 1709–1712
51. Csere, P., Lill, R., and Kispal, G. (1998) FEBS Lett. 441, 266–270
52. Radisky, D. C., Babcock, M. C., and Kaplan, J. (1999) J. Biol. Chem. 274, 4497–4499
53. Harris, Z. L., Durley, A. P., Man, T. K., and Gitlin, J. D. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10812–10817
54. Fleming, R. E., Migas, M. C., Zhou, X. Y., Jiang, J., Britton, R. S., Brunt, E. M., Tomatsu, S., Wahred, A., Bacon, B. R., and Sly, W. S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3143–3148