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Balance between Endogenous Superoxide Stress and Antioxidant Defenses

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Cells devoid of cytosolic superoxide dismutase (SOD) suffer enzyme inactivation, growth deficiencies, and DNA damage. It has been proposed that the scant superoxide (O_2^-) generated by aerobic metabolism harms even cells that contain abundant SOD. However, this idea has been difficult to test. To determine the amount of O_2^- that is needed to cause these defects, we modulated the O_2^- concentration inside *Escherichia coli* by controlling the expression of SOD. An increase in O_2^- of more than twofold above wild-type levels substantially diminished the activity of labile dehydratases, an increase in O_2^- of any more than fourfold measurably impaired growth, and a fivefold increase in O_2^- sensitized cells to DNA damage. These results indicate that *E. coli* constitutively synthesizes just enough SOD to defend biomolecules against endogenous O_2^- so that modest increases in O_2^- concentration diminish cell fitness. This conclusion is in excellent agreement with quantitative predictions based upon previously determined rates of intracellular O_2^- production, O_2^- dismutation, dehydratase inactivation, and enzyme repair. The vulnerability of bacteria to increased intracellular O_2^- explains the widespread use of superoxide-producing drugs as bactericidal weapons in nature. *E. coli* responds to such drugs by inducing the SoxRS regulon, which positively regulates synthesis of SOD and other defensive proteins. However, even toxic amounts of endogenous O_2^- did not activate SoxR, and SoxR activation by paraquat was not at all inhibited by excess SOD. Therefore, in responding to redox-cycling drugs, SoxR senses some signal other than O_2^-.

The discovery of superoxide dismutase (SOD) was accidental (51), and it has been a long road to an understanding of its role in cell physiology. Its wide distribution among aerobic organisms (50) suggested both that superoxide (O_2^-) is formed inside all cells that grow in air and that O_2^- is toxic. This hypothesis has been extended to predict that enough O_2^- might evade SOD to generate chronic oxidative damage, the gradual accumulation of which may contribute to age-associated pathologies (2, 7, 11, 15). However, the physiological role of SOD was actively debated for many years because it was difficult to discern intracellular sources and targets of O_2^-.

The construction of *Escherichia coli* mutants lacking the cytosolic SODs gave the first insight into the significance of intracellular O_2^- (6). These mutants exhibit several defects when grown aerobically: they are auxotrophic for branched-chain, aromatic, and sulfur-containing amino acids, and they catabolize only fermentable carbon sources (6, 35). The SOD$^-$ mutants also show high rates of spontaneous mutagenesis (12). Severe phenotypes of SOD$^-$ mutants, ranging from growth defects to decreased fertility and life spans, were subsequently observed in higher organisms as well (56, 65).

Analysis of the *E. coli* mutants illuminated the molecular targets of O_2^-. The branched-chain auxotrophy and the requirement for a fermentable carbon source arise because O_2^- inactivates dihydroxyacid dehydratase (13, 41), aconitase (20), and fumarases A and B (14, 46). These dehydratases, as well as 6-phosphogluconate dehydratase of *E. coli* (18) and similar enzymes of other organisms (14, 16, 31, 32), each contain a distinctive [4Fe-4S] cluster that provides a local positive charge to help bind and dehydrate the substrate. In the absence of substrate, O_2^- can oxidize and thereby destabilize the exposed cluster. Iron then dissociates, resulting in the loss of enzyme activity. Reactivation of the clusters occurs in vivo, and it is likely that labile enzymes are repeatedly damaged and repaired during oxidative stress, so that the steady-state activity is a balance of the two processes. When iron from the damaged cluster spills into the cytosol, it is available to participate in Fenton chemistry (38, 47) and catalyzes oxidative damage to DNA (40). This causes the high rate of mutagenesis that characterizes SOD mutants.

An important question is whether the O_2^- production during aerobic metabolism is sufficient to cause damage in a cell that contains SOD. Unfortunately, the intracellular O_2^- concentration of either SOD-proficient or SOD-deficient cells is below detection by current techniques capable of measuring O_2^- (34, 43). Flavoproteins of the electron transport chain generate an estimated 3 μM O_2^-/s during exponential growth (34). Yet *E. coli* contains ca. 3,000 U of SOD per ml, enough to restrict the calculated steady-state O_2^- concentration to 10^{-10} M, or about 0.1 molecule per cell. In fact, the concentration of SOD exceeds that of O_2^- in vivo by about 100,000 to 1, which is an unprecedented relation between enzyme and substrate (1). Thus, it may seem unlikely that such large amounts of SOD are necessary to prevent damage from endogenous O_2^- sources.

An alternative is that *E. coli* synthesizes so much SOD solely as a preemptive defense against the O_2^- that is produced during exposures to redox-cycling drugs, many of which are made by other microorganisms as a means to defend their habitat. Redox-cycling drugs are able to enter bacterial cells and generate O_2^- through interactions with flavoproteins. The rate of O_2^- production by redox-cycling drugs can approach the rate of respiration, exceeding the normal rate of endogenous O_2^- formation by orders of magnitude (28, 33). *E. coli* mutants that are deficient in SOD activity are hypersensitive to these drugs (6, 33).

SOD synthesis is positively regulated by the SoxRS regulon

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TABLE 1. Strains, plasmids, and phage

Strain, plasmid, or phage	Relevant genotype	Source or reference
Strains		
AB1157	F^- *thr-1 leuB6 proA2 his-4 thi-1 argE2 lacY1 galK2 rpsL surE44 ara-14 xyl-15 mtl-1 tsx-33*	37
AS240	Same as that for AB1157 plus (*sodA*::Mu d PR13)*25* (*sodB-kan*)*1-Δ2 zdg-299*::Tn*10* λAS2	This study
AS241	Same as that for AS240 plus pCKR101	This study
AS290	Same as that for AB1157 plus pCKR101	This study
AS291	Same as that for JI132 plus pCKR101	This study
AS356	Same as that for JI131 plus pCKR101	This study
AS357	Same as that for JI130 plus pCKR101	This study
AS358	Same as that for TN530 plus (*sodA*::Mu d PR13)*25* (*sodB-kan*)*1-Δ2*	This study
AS360	Same as that for AS358 plus pDT1.16	This study
AS364	Same as that for TN530 plus pDT1.16	This study
AS370	Same as that for AS240 plus pMS421	This study
AS372	Same as that for JI131 plus pMS421	This study
AS374	Same as that for AB1157 plus pMS421	This study
AS376	Same as that for JI130 plus pMS21	This study
AS395	Same as that for TN530 plus (*sodA*::Mu d PR13)*25*	This study
AS396	Same as that for AS395 plus pKK1	This study
JI130	Same as that for AB1157 plus (*sodA*::Mu d PR13)*25*	37
JI131	Same as that for AB1157 plus (*sodB-kan*)*1-Δ2*	37
JI132	Same as that for AB1157 plus (*sodA*::Mu d PR13)*25* (*sodB-kan*)*1-Δ2*	37
TN530	λ *soxS*::*lacZ* Δ*soxR* Δ*lacU169 rpsL*	55
Plasmids		
pCKR101	pBR328 derivative containing *lacI*^{q}	Jeff Gardner
pMS421	pSC101 derivative containing *lacI*^{q}	24
pDT1.16	pBR322 derivative containing *tac* promoter fused to *sodA*	63
pKK1	pBR328 derivative containing *sodB*	38
Phage λAS2	*lacA lacY*′ P*tac-sodA kan*	This study

(25, 64), which responds to redox-cycling drugs. This raised the question of whether the signal to which SoxRS responds is O_2^- itself or some other effect of the drug (18, 22, 44, 48, 54). If the signal were O_2^- itself, then it is possible that the SoxRS regulon also responds to metabolically generated O_2^- levels, adjusting SOD synthesis in order to keep the intracellular O_2^- concentration within a narrow range. Such an adaptive mechanism has been proposed for the OxyR-dependent induction of catalase in response to endogenous hydrogen peroxide (23).

To determine whether abundant SOD is needed to defend cells against damage by endogenous O_2^-, we constructed a strain in which the cytosolic SOD activity could be modulated. The extent of O_2^- damage was then assessed over a range of SOD concentrations. The analysis of our results will lead us to conclusions regarding the ability of cells to tolerate increases in O_2^-.

MATERIALS AND METHODS

Reagents. Manganese (II) chloride tetrahydrate was obtained from Aldrich Chemical Company, Inc., Milwaukee, Wis. Beef liver catalase was purchased from Boehringer Mannheim, Indianapolis, Ind. Coomassie protein assay reagent was obtained from Pierce, Rockford, Ill. All other chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. Water used in all reagents was from the house deionized system and was further purified by a Labconco Water Pro PS system to minimize metal contamination.

Strain construction. The strains that were used in this study are shown in Table 1. During strain construction, the introduction of chromosomal null mutations was achieved by P1 transduction with selection for linked antibiotic resistance markers.

To construct a strain in which the expression of SOD could be modulated, the *Eco*RI/*Msc*I fragment from pDT1.16 (63) containing *sodA* under control of the *tac* promoter was cloned into the vector pRS551 (61), which had been digested with *Eco*RI and *Sna*BI. The resultant plasmid was designated pAS1. The regions of pAS1 which flank the *sodA* insert have homology to λRS45 and permitted marker exchange onto the λ phage (61). The insert on this phage is preceded by four terminator sequences, preventing transcriptional readthrough from upstream genes. Single-copy lysogens were recovered in *E. coli* by selection for phage-encoded kanamycin resistance. Null mutations of *sodA* and *sodB* were introduced into the lysogen by transduction. Thus, synthesis of cytosolic SOD was under the control of the *tac* promoter and responded to the addition of isopropyl-β-D-thiogalactopyranoside (IPTG). The presence of the *lacY1* mutation in all these strains circumvented the difficulties that occur when a titrating molecule induces synthesis of its own transporter (53, 60).

Media and cell growth. Defined media contained minimal A salts and either 0.2% glucose, 0.2% gluconate, or 40 mM fumarate as a carbon source. Histidine, leucine, threonine, arginine, and proline were present in all media to satisfy the genetic auxotrophies of AB1157 derivatives; supplemented media additionally contained either a 0.5 mM concentration of the other 15 amino acids or 0.25% Casamino Acids. Luria broth (LB) was used for some experiments. pCKR101 was maintained with 50 μg of ampicillin per ml, and pMS421 was maintained with 100 μg of spectinomycin per ml and 50 μg of streptomycin per ml.

The same media were used for preliminary anaerobic cultures as were used in the aerobic portions of the experiments, except when cultures were grown in fumarate medium. For the experiments with fumarate medium, cells were first cultured anaerobically and then were cultured aerobically in minimal glucose medium before being washed and diluted into fumarate medium. Overnight cultures of SOD-deficient strains were always incubated in a Coy anaerobic chamber (85% N_2, 10% H_2, 5% CO_2) to prevent the accumulation of phenotypically suppressed mutants. The stationary-phase cultures were typically diluted to an optical density at 600 nm (OD_{600}) of 0.010 in anaerobic medium and cultured for at least four generations before being diluted again to an OD_{600} of 0.010 in a series of flasks containing 300 to 500 ml of aerobic media. The flasks were supplemented with a range of concentrations of IPTG (typically 5 to 50 μM) and $MnCl_2$ (0.1 to 1 μM) to vary manganese SOD (MnSOD) activity. Cell density was monitored by absorbance during growth to an OD_{600} of 0.1 to 0.2. At an OD_{600} of 0.10, portions of the cultures were harvested for SOD and enzyme assays. The growth rates that are reported were determined by using density measurements made before and after some cells had been harvested for SOD assays. Some imprecision in the correlation between growth rate and SOD activity may have occurred due to a slight drift in SOD content during the period of growth measurements.

Assays of labile enzymes. The lysis buffer used for aconitase assays contained 50 mM Tris (pH 7.4), 0.6 mM $MnCl_2$, and 20 μM fluorocitrate; that for 6-phosphogluconate dehydratase assays contained 50 mM Tris buffer (pH 7.6); and that for fumarase assays contained 50 mM potassium phosphate buffer (pH 7.8). Exponentially growing cultures were centrifuged at 15,000 × *g* for 3 min, resuspended in 1 ml of buffer, and lysed by either sonication or passage through a

French pressure cell without any difference in the results. The lysates were clarified by centrifugation in a Fisher Scientific microcentrifuge for 1 min at 12,000 rpm and immediately frozen in a dry ice-ethanol bath to avoid loss of enzyme activity before assay. After the lysates were thawed, aconitase assays and 6-phosphogluconate assays were performed (19, 20). Fumarase was assayed by monitoring the conversion of 50 mM L-malate to fumarate at an OD of 250 nm ($\varepsilon_{[fumarate]} = 1.62$ mM^{-1} cm^{-1}) in sodium phosphate buffer (pH 7.3). Superoxide-resistant fumarase C activity was measured after fumarase A activity had been inactivated by O_2^-. To this end, 1 ml of diluted extract was incubated with 2.8 mU of bovine xanthine oxidase and 55 μM xanthine for 15 min at room temperature. L-Malate was then added, and the sample was assayed.

Reactivation of aconitase was achieved in cell suspensions prior to lysis. Tetracycline (100 μg/ml) was added to aerobic cultures to block protein synthesis, the cells were centrifuged, and the cell pellets were transferred into the anaerobic chamber. There, the pellets were resuspended in anaerobic lysis buffer containing 100 μg of tetracycline per ml. At time points, aliquots of the cell suspension were removed from the chamber, lysed, clarified, and frozen as detailed above. The assays were performed as described previously (20). Control experiments confirmed that inactive enzymes were not detectably reactivated during their few moments in anaerobic cell pellets nor were active enzymes inactivated during storage prior to assay (data not shown).

Other methods. Induction of SoxRS was achieved by the addition of the indicated concentration of paraquat followed by a 45-min incubation at 37°C. Assays for β-galactosidase were done as described previously (52). SOD activity was assayed after overnight dialysis against 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA (51). Protein assays were performed with chicken egg albumin as a protein standard and Coomassie protein assay reagent.

Sensitivity to killing by H_2O_2 was determined by using cultures growing exponentially in glucose-Casamino Acids medium ($OD_{600} = 0.10$). Hydrogen peroxide was added to a final concentration of 2.5 mM, and the cultures were shaken for 10 min at 37°C. Dilutions were made into LB containing catalase (130 U/ml) and plated. Colonies were counted after a 16-h incubation, and the rate of killing (k) was calculated by the equation $k = 1/t \times \ln(N_0/N_1)$, where N_0 and N_1 are the initial and final numbers of viable cells, respectively, and t is time.

Measurements of free iron were made by electron paramagnetic resonance (EPR) analysis (40), using exponentially growing cultures ($OD_{600} = 0.10$) in 2 liters of aerobic glucose-Casamino Acids medium. Iron levels were quantitated by normalizing the amplitude of the iron signal to iron standards, and internal concentrations were calculated by using the intracellular volume (34).

RESULTS

Construction of the experimental strain. To determine the amount of cytosolic SOD that is sufficient to prevent oxidative damage, we constructed an *E. coli* strain in which the amount of SOD could be modulated. The major challenge which we anticipated was the need to minimize SOD expression sufficiently so that phenotypes of oxidative injury could emerge, and therefore it was important to reduce the construct to a single copy. We subcloned a P*tac-sodA* insert from pDT1.16 onto plasmid pRS551 and allowed it to recombine onto a lambda phage (see Material and Methods). The lambda phage was then used to infect a SOD-proficient strain, and a lysogen in which the lambda had stably integrated into the chromosome was isolated. Chromosomal null mutations in *sodA* and *sodB* were introduced by P1 transduction. The resulting lysogen was then transformed with either pCKR101 or pMS421, medium-copy-number plasmids that overexpressed the *lac* repressor and further reduced the basal expression of the *sodA* gene to approximately 3% of that of the wild type.

In this background, MnSOD, the sole cytosolic SOD, could be induced by the administration of IPTG in the presence of Mn^{2+}. The concentration of manganese added was kept below 1 μM to prevent SOD-independent dismutation of O_2^- (8), which became significant at higher Mn^{2+} concentrations (data not shown). The level of maximum induction with IPTG and this manganese supplementation was roughly 30% the activity of a wild-type strain; however, since phenotypes are apparent only at lower SOD titers (see below), this level of expression was sufficient for this work. Strains that retained either the chromosomal *sodA* or *sodB* genes were also constructed. In most media, these single mutants contained >30% of wild-type SOD activity and exhibited wild-type behavior. The lone exception was the *sodB* mutant grown in fumarate, because the *sodA* gene is poorly expressed in fumarate medium (see below).

Inhibition of growth by endogenous superoxide. Strains of *E. coli* that lack both cytosolic SODs cannot grow in minimal medium without amino acid supplements (6, 35). Cells containing 25% of the wild-type SOD level grew at 75% of the rate measured for wild-type cells in unsupplemented medium (Fig. 1A). Cells containing less than 10% grew at rates that approached zero.

SOD mutants require branched-chain, aromatic, and sulfurous amino acids. At limiting SOD concentrations, the separate addition of these amino acid groups was unable to restore rapid growth (data not shown). However, when media were supplemented with all 20 amino acids, cultures grew as rapidly as wild-type cells unless SOD levels were reduced to less than 10% of the wild-type level, and even then growth continued at approximately 60% of the rate of wild-type cells (Fig. 1B). Therefore, the decreased growth rate of the unsupplemented cells occurred primarily because of damage to multiple amino acid biosynthetic pathways, whereas a less-sensitive, unknown target limited the growth rate of supplemented cells.

Inactivation of [4Fe-4S] dehydratases. The effects of O_2^- can be more precisely observed by measuring the activities of the enzymes that it directly damages. We assayed aconitase and 6-phosphogluconate dehydratase over a range of SOD levels. Cultures were grown with amino acid supplements and fermentable carbon sources so that low SOD activities had only minor effects on growth rates and presumably on the rates of metabolic O_2^- production.

The data are shown in Fig. 2. Both aconitase and 6-phosphogluconate dehydratase activities were progressively lower in cells containing less than 40% of wild-type SOD activity. At very low SOD activities, the 6-phosphogluconate dehydratase activity approached zero, whereas about 10% of the wild-type aconitase activity remained active. Gruer et al. have reported that *E. coli* contains two or three aconitase isozymes (26, 27); we are investigating the possibility that a minor isozyme is resistant to O_2^-.

It seemed possible, albeit unlikely, that the low dehydratase activities in SOD-deficient cells reflected a lower rate of synthesis rather than enzyme damage. To determine whether the SOD-deficient cells contained inactivated dehydratases, we added inhibitors of protein synthesis to a fraction of the SOD-deficient cells and incubated them under anaerobic conditions prior to harvesting. The cluster repair processes that are active during such an incubation restored the aconitase activity to that of a wild-type strain within 10 min (data not shown). Thus, the low dehydratase activities of SOD-limited cells accurately reflect enzyme damage.

These results indicate that *E. coli* requires nearly its normal complement of SOD to prevent growth deficiencies from endogenous O_2^-. Furthermore, it is possible that the negative effect of O_2^- may have been underestimated due to a reduction in the metabolic O_2^- production as the growth rate declined.

Effect of superoxide on DNA damage and free iron. The oxidation of [4Fe-4S] dehydratase clusters by O_2^- causes the release of iron into the cell cytoplasm. This free iron can catalyze the Fenton reaction and thereby rapidly generate DNA damage in SOD^- mutants. The vulnerability to DNA damage is reflected by a high rate of killing when SOD-deficient cells are exposed to exogenous hydrogen peroxide (37). Sensitivity to hydrogen peroxide increased greatly when cells contained less than 15% the wild-type level of SOD (Fig. 3A). This dosimetry agreed with the sensitivity of the dehydratases to decreases in SOD (Fig. 2).

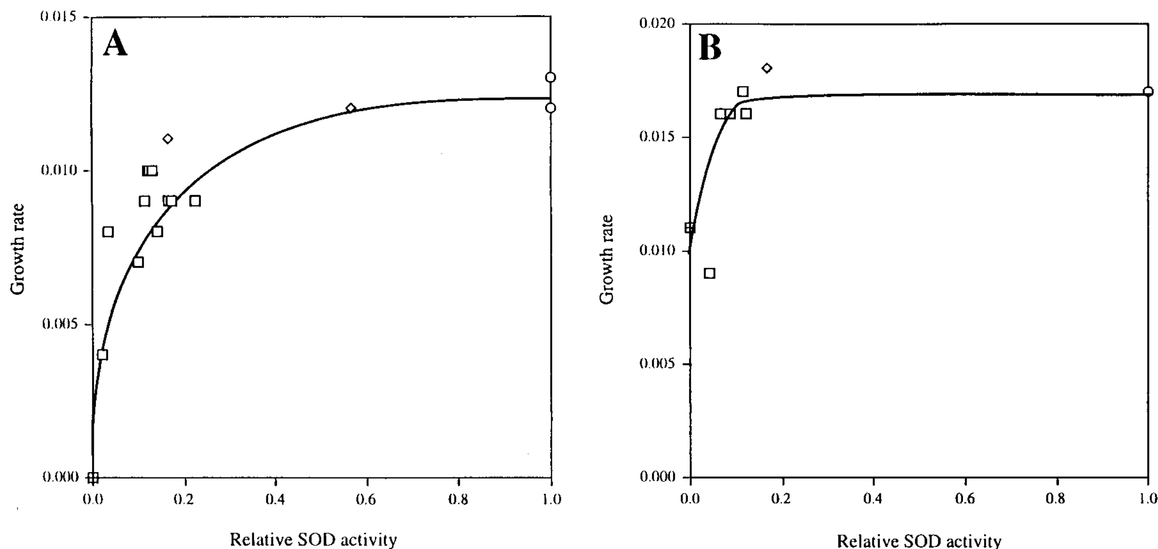

FIG. 1. Effect of SOD activity on growth rate in minimal medium. Cultures of strains AS290 (SOD^+) (○), AS356 (*sodB*) (◇), AS291 (*sodA sodB*) (⊞), and AS241 (*sodA sodB* λ P*tac-sodA*) (□) were grown in unsupplemented (A) or amino acid-supplemented (B) minimal A medium containing glucose. SOD activity in AS241 was modulated with a range of both IPTG and $MnCl_2$ concentrations as described in Materials and Methods. The data shown for growth in unsupplemented minimal medium is a combination of the data from two separate experiments, with the relative SOD activity normalized to 9.2 U/mg, the average specific activity found in wild-type cells. SOD activities for the cultures grown in supplemented minimal medium were normalized to the specific activity of the wild-type culture, 5.8 U/mg.

To verify that this heightened sensitivity reflected a change in iron homeostasis, we used a whole-cell EPR technique to measure the internal concentration of free iron (Fig. 3B). An SOD-proficient strain contained only a modest amount of free iron (6 μM), while a strain devoid of SOD contained almost 10-fold more (52 μM), consistent with a previous report (40). Cells that expressed 6% of the wild-type level of SOD activity had substantially more free iron than did SOD-proficient cells (25 μM), which readily accounts for the DNA damage data (Fig. 3A).

It is striking that the rate of DNA damage was not reduced to zero as SOD levels increased. Apparently, even unstressed *E. coli* contains a pool of free iron, and so it is only when the flux of iron from damaged dehydratases is large that O_2^- has an appreciable effect on the pool size. Because of that basal pool of iron, *E. coli* could not fully avoid oxidative DNA damage by producing higher amounts of SOD. In fact, SOD overproduction has been shown to have no effect upon oxidative DNA damage in a wild-type cell (38).

The SoxRS regulon is induced by drugs but not by superoxide. Since the cell makes just enough SOD to protect itself from metabolic O_2^-, it seemed plausible that the SOD con-

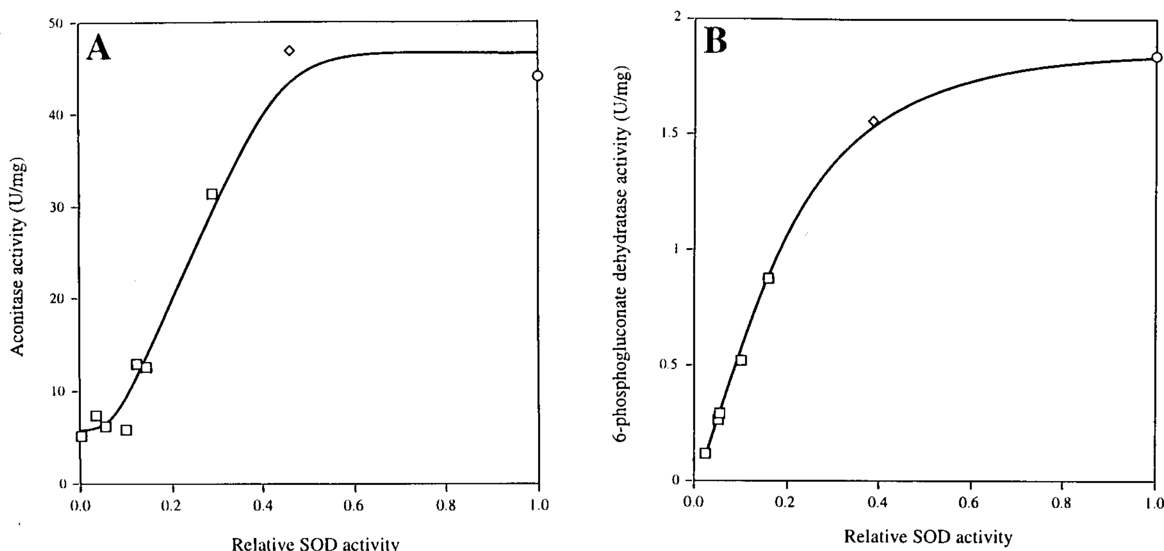

FIG. 2. Effect of SOD activity on aconitase and 6-phosphogluconate dehydratase activities. Cells for aconitase (A) and 6-phosphogluconate dehydratase (B) assays were grown in minimal medium containing Casamino Acids and either glucose or gluconate, respectively. SOD activities were modulated and extracts were prepared as detailed in Materials and Methods. The relative SOD activity is normalized to wild-type specific activity. The wild-type SOD activity in glucose medium was 6.1 U/mg; in gluconate medium, it was 9.8 U/mg. For identification of symbols, see the legend to Fig. 1.

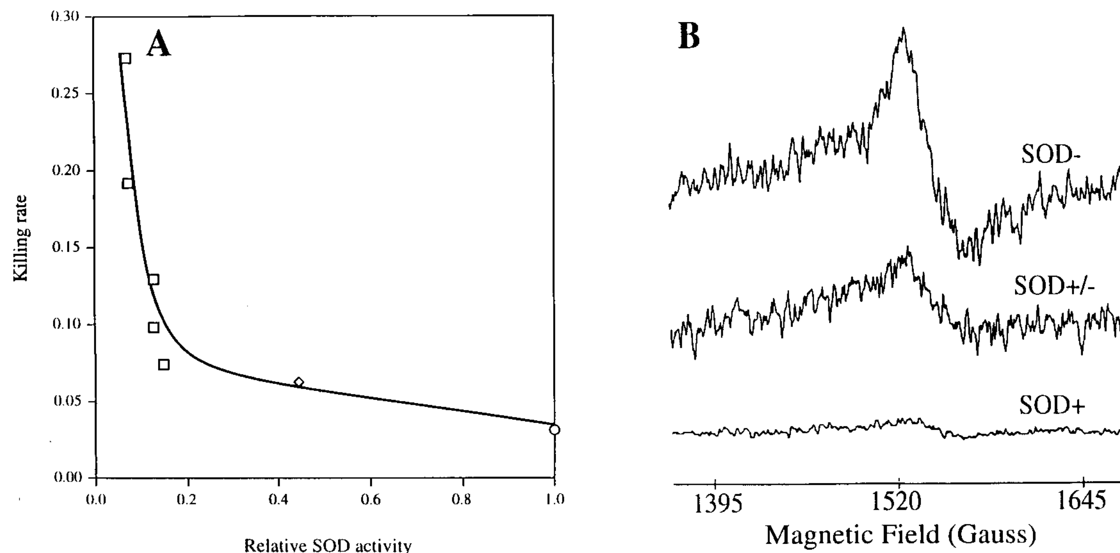

FIG. 3. Sensitivity to DNA damage and detection of internal free iron. (A) Rates of killing were determined during exposure to 2.5 mM hydrogen peroxide. Cell death is due to DNA damage (36). Cultures were grown and challenged in minimal medium containing Casamino Acids and glucose. A value of 8.0 U of specific SOD activity per mg for the SOD^+ strain was used to normalize the SOD activities. (B) The EPR spectra are shown for a SOD^+ strain, a *sodA sodB* (SOD^-) strain, and a *sodA sodB* strain containing λ P*tac-sodA* (SOD+/−) without further induction by IPTG. The peak at *g* equal to 4.3 is from Fe^{3+} complexed to deferoxamine mesylate, a chelator of free iron. For identification of symbols, see the legend to Fig. 1.

centration might be continually adjusted in response to O_2^- levels. The SoxRS regulon, which controls MnSOD synthesis, has been proposed to respond directly to O_2^- and is efficiently induced by redox-cycling drugs that generate O_2^-. The activated SoxR protein induces synthesis of SoxS (55, 67), which in turn positively regulates a set of genes whose products may be protective, including *sodA*. If SoxRS were to modulate SOD in response to O_2^-, then the regulon should be highly induced in SOD-deficient strains. Using strains containing *soxS*::*lacZ* fusions on a lambda prophage, we assayed β-galactosidase to monitor the degree of SoxRS induction in wild-type strains and in SOD mutants (Fig. 4). The striking result was that *soxS* was not induced in SOD mutants, despite the fact that they contained enough O_2^- to completely inactivate metabolic pathways. Glucose-6-phosphate dehydrogenase, a member of the SoxRS regulon, was also minimally induced in SOD mutants (data not shown). In contrast, *soxS* was highly expressed when cells were exposed to paraquat (Fig. 4).

We considered the possibility that O_2^- had failed to induce the regulon because the SOD mutants had been necessarily cultured in medium that did not require the function of superoxide-sensitive enzymes. That is, SoxRS might only be activated when the enzymes which it defends are essential for growth. Therefore, we monitored the expression of fumarase C, a member of the regulon (45), in cells that contained limiting amounts of SOD during growth in fumarate medium. Fumarase function is essential for the catabolism of fumarate. Fumarase A, the major fumarase activity of aerobic cells (66), utilizes a [4Fe-4S] cluster that O_2^- rapidly inactivates (14, 46). The gene encoding fumarase B is expressed only under anaerobic conditions; we observed that a *fumA fumC* mutant had less than 3% of the normal fumarase activity when grown aerobically (data not shown). Fumarase C is a minor isozyme that has no iron-sulfur cluster. It is resistant to O_2^- and it is induced by SoxRS.

The growth rate in fumarate medium declined when cells contained less than 20% of the wild-type SOD activity (Fig. 5A). Fumarase A activity fell in parallel (Fig. 5B). Importantly, as fumarase A activity declined, fumarase C activity increased only slightly. Whether SoxRS activation is responsible for the modest induction of fumarase C was not tested. When SOD activity was limited to 10% of the wild-type level, fumarase A was 80% inactive and fumarase C was induced only twofold. This was not enough to restore total fumarase activity or to

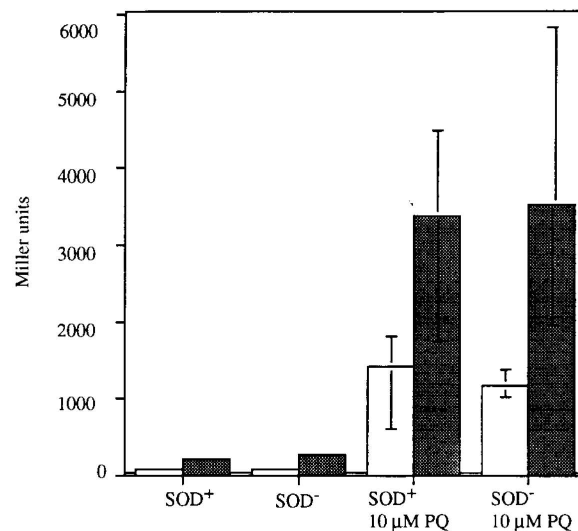

FIG. 4. Endogenous O_2^- is a poor inducer of SoxRS. SOD-proficient and SOD-deficient lysogens of a λ containing a *soxS*::*lacZ* fusion were assayed for β-galactosidase activity in minimal medium containing Casamino Acids and glucose (shaded bars) and in LB medium (open bars). Cultures of each strain were grown to an OD near 0.050. The cultures were each split into two flasks, paraquat was added to one to a final concentration of 10 μM, and all cultures were incubated for an additional 45 min. Cells grown in LB were washed and resuspended in minimal medium containing Casamino Acids and glucose prior to assay. The data shown have been normalized to the β-galactosidase activity present in the uninduced cultures. The error bars represent the ranges of activity measured for four independent cultures.

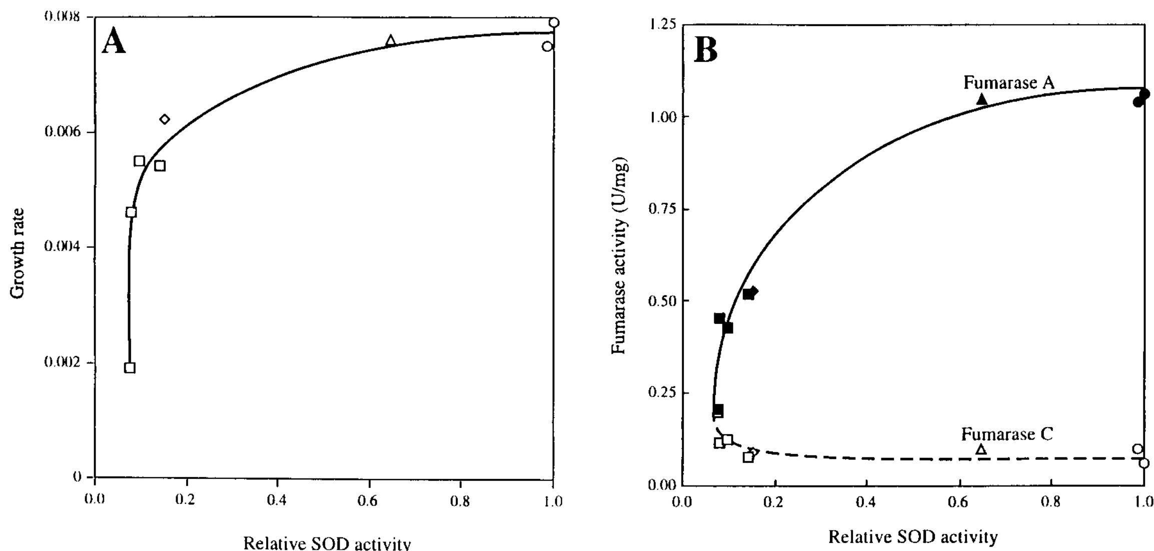

FIG. 5. Growth rates (A) and fumarase activities (B) of cultures with modulated SOD activity. Cultures of strains AS370 (*sodA sodB* λ P*tac-sodA*) (□, ■), AS372 (SOD^+) (○, ●), AS374 (*sodB*) (◇, ◆), and AS376 (*sodA*) (△, ▲) were grown aerobically in unsupplemented minimal medium containing fumarate. The relative SOD activity was determined by normalizing to a wild-type activity of 5.2 U/mg.

permit normal growth in the medium. In contrast, paraquat was a much more effective inducer: at a dose that generated enough O_2^- (in a SOD-proficient strain) to inactivate fumarase A by 80%, it induced *soxS* expression 11-fold (Table 2). This induction, which is mediated by SoxRS (45), was sufficient to maintain normal total fumarase activity. The implication was that SoxR does not sense toxic levels of O_2^- but does sense some other signal that is provided by the paraquat.

To test this idea more directly, we examined whether excess SOD could inhibit *soxS* induction by paraquat. The *soxS*::*lacZ* fusion was placed into a strain expressing only *sodB* (to avoid the complications of *sodA* induction) and then that strain was transformed with a plasmid bearing *sodB*. There was no difference in the induction profiles of the two strains despite a 20-fold difference in SOD activities (Fig. 6). If O_2^- were the inducer, one would have expected the half-inducing dose to be 20 times higher in the overproducer. Similar paraquat treatment was unable to induce *soxS* in a *soxR* deletion mutant (data not shown), confirming that in paraquat-treated cells *soxS* induction responds exclusively to SoxR. These experiments collectively demonstrated that O_2^- is neither sufficient nor necessary for SoxRS induction.

DISCUSSION

Our results show that *E. coli* can tolerate only small decreases in SOD content. A decrease in SOD of more than twofold led to significant dehydratase inactivation, and further

TABLE 2. Paraquat induction of fumarase C compensates for the inactivation of fumarase A

Cell culture	Activity (U/mg)		
	Fumarase A	Fumarase C	Total fumarase
SOD^+	1.27	0.09	1.36
SOD^+ + 10 μM paraquat	0.24	0.94	1.18
10% of Wild-type SOD	0.20	0.19	0.39

decreases in SOD both lowered the growth rate in unsupplemented medium and resulted in sensitivity to DNA damage. Clearly, enough O_2^- is made during normal metabolism to require the synthesis of abundant SOD.

For wild-type cells, it is useful to determine the extent by which O_2^- production must increase before toxicity results. The answer can be inferred from this study if the steady-state O_2^- concentration varies inversely with SOD activity. That has been generally assumed to be true (17, 34) because SOD functions as a first-order, unsaturable enzyme at physiological concentrations of O_2^- (4, 5, 57) and because no other significant

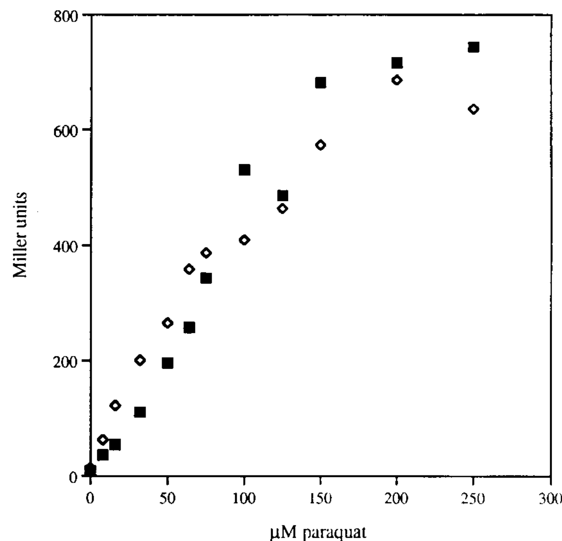

FIG. 6. Paraquat induction of SoxRS does not require O_2^-. Strains AS395 (*sodA* λ*soxS*::*lacZ*) (◇) and AS396 (*sodA* λ*soxS*::*lacZ* p*sodB*) (■) were grown aerobically in LB medium to an OD of 0.050. The culture was aliquoted into tubes containing paraquat and further incubated at 37°C for 45 min. The data were normalized to the activity present in the uninduced cultures (Miller units). SOD activity was assayed, with AS395 having 9 U/mg and AS396 having 216 U/mg.

scavenger of O_2^- has been found in the cytosol of *E. coli* (35). Although O_2^- can spontaneously dismute, it will do so at an appreciable rate only in cells containing <0.1% of wild-type SOD activity (calculated from reference 34). Labile iron sulfur clusters are too scarce (100 μM) and too slowly reactivated (half-life [$t_{1/2}$] = 7 min) (39) to consume more than 5% of the 200 μM O_2^- flux/min. The absence of SOD-independent scavenging mechanisms is supported by the fact that O_2^- toxicity continues to worsen when even very low SOD activities are further diminished (Fig. 2).

Therefore, during steady-state conditions, the formation of O_2^- is balanced by its dismutation by SOD. The following equations describe the relation between O_2^- concentration and SOD concentration, where k_f and k_{SOD} denote the rate of O_2^- formation and the rate constant for O_2^- dismutation by SOD, respectively:

$$k_f = k_{SOD} \times [SOD] \times [O_2^-] \quad (1)$$

$$[O_2^-] = (k_f/k_{SOD}) \times [SOD]^{-1} \quad (2)$$

Thus, the intracellular O_2^- concentration varies inversely with the amount of SOD. A threefold decrease in SOD results in a threefold increase in the steady-state O_2^- concentration. Notably, this increase could also result from a threefold increase in the rate of O_2^- formation. Even if the assumption that most O_2^- were scavenged by SOD were wrong, the consequence would be that our estimate of O_2^- sensitivity would be too conservative. In that circumstance, an additional term would be included in the denominator of equation 2, so that the toxic effects of O_2^- would ensue from less than a threefold increase in its formation.

By using the relation between SOD and O_2^- concentrations, the data can be reevaluated. Substantial enzyme damage will result when O_2^- levels increase by more than twofold, and growth deficits will become pronounced upon an increase of more than fourfold. In fact, because the measurements of growth rates were less exacting than assays of dehydratase activity, we suspect that more-precise measurements might indicate that growth declines at O_2^- concentrations even closer to those of wild-type cells. Clearly, *E. coli* is poised near the brink of toxicity from endogenous oxidants. This fact is remarkable given the extremely low concentration of O_2^- in living cells and attests to both the high rate and specificity of its reactions with dehydratase iron-sulfur clusters. It is also clear that additional O_2^- formation by redox-cycling drugs would be detrimental if SOD synthesis were not augmented.

Concordance between calculated and observed enzyme damage. The fractional activity of a dehydratase population within the cell is determined by the balance between the rate of intracellular O_2^- formation and the rate constant for O_2^- inactivation of the dehydratase, on the one hand, and the concentration of SOD and the speed of enzyme reactivation, on the other hand. These four parameters have all been measured independently, making it possible to test whether they fit our results.

Superoxide is formed primarily in *E. coli* by the reaction between oxygen and reduced components of the respiratory chain. In air-saturated minimal medium, the rate was estimated to be 3 μM O_2^-/s (34). Based on the SOD content of these cells, the steady-state O_2^- concentration was calculated to be 2×10^{-10} M. It has since been shown that only half of the electron flux passes through the auto-oxidizable NADH dehydrogenase, which lowers the best estimate of O_2^- concentration to 10^{-10} M (unpublished data).

The rate constants for the inactivation by O_2^- of several dehydratases, namely, fumarase A, fumarase B, dihydroxyacid dehydratase, and beef heart aconitase, were each determined in vitro to lie between 1×10^6 and 6×10^6 M^{-1} s^{-1} (14). These rates were measured by two indirect techniques that produced similar but not equivalent results. A higher value has been reported for purified *E. coli* aconitase, 3×10^7 M^{-1} s^{-1} (29). In all studies, the inactivation rates were lowered somewhat by the presence of substrate (14, 20), which partially shields the active site; this might particularly affect aconitase, since substrate more effectively protects aconitase and since citrate levels may be saturating in vivo (20, 42, 49). Based on the values determined by Flint, we use 3×10^6 M^{-1} s^{-1} as a representative value for the inactivation rate constants ($k_{inactivation}$) of the dehydratases in the following equation:

$$-dE/dt = k_{inactivation} \times E \times [O_2^-] \quad (3)$$

where E is the amount of active enzyme. By integration where $E1$ and $E2$ represent amounts of active enzyme at times separated by an interval (+), we calculate

$$\ln(E1/E2) = (3 \times 10^6\ M^{-1}\ s^{-1})(10^{-10}\ M)t$$

giving $t_{1/2}$ = 39 min. These calculations predict that the labile enzymes of *E. coli* are likely to be damaged at least once per generation (55 min) in glucose-saturated medium. The dehydratases are likely to cycle between active and inactive forms. Damage to the enzymes may occur even more often per generation when *E. coli* is in its natural habitat, which supports a much lower growth rate.

Under steady-state conditions, this inactivation rate will be equal to the reactivation rate. The reactivation half-time was measured to be 7 min ($k_{reactivation}$ = 0.00165 s^{-1}) (39):

$$k_{inactivation} \times E \times [O_2^-] = k_{reactivation} \times (T - E) \quad (4)$$

where T is the total (active plus inactive) enzyme. In a wild-type strain,

$$E \times (3 \times 10^6\ M^{-1}\ s^{-1})(10^{-10}\ M) = (0.00165\ s^{-1})(T - E)$$

giving E/T = 0.85. Therefore, SOD-proficient cells growing in air-saturated medium are expected to contain enough O_2^- that the labile dehydratases are only 85% active. This agrees with the observation that aconitase activity increased by about 13% when aerobic cells were shifted to anaerobic conditions in the presence of protein synthesis inhibitors (20). Comparisons of the amounts of SOD and labile clusters inside cells (12 and 100 μM, respectively) and of their rate constants for reaction with O_2^- (2×10^9 and 3×10^6 M^{-1} s^{-1}) suggest that SOD scavenges about 99% of the O_2^- before it damages dehydratases.

More generally, one can predict the fraction of active enzyme as a function of SOD content. Using equation 4, we calculate

$$E \times (3 \times 10^6\ M^{-1}\ s^{-1})(10^{-10}\ M) \times f_{SOD}^{-1} = 0.00165\ s^{-1} \times (T - E) \quad (5)$$

where f_{SOD} is the SOD activity as a fraction of that found in wild-type cells, and

$$E/T = \{1 + [1/(5.5 \times f_{SOD})]\}^{-1} \quad (6)$$

Consequently, dehydratases would be half inactive when SODs were present at 20% of the wild-type activity. This result is in good agreement with Fig. 2 and 5. Because the values used in this calculation were approximations, the concordance seen between the calculated and observed behaviors should not be considered precise. Nevertheless, the in vivo results support the published in vitro measurements of O_2^- formation and of

dehydratase sensitivity. Apparently, the field has achieved a detailed, coherent view of the physiology of oxidative stress. It seems that *E. coli* has evolved so that the production of O_2^- and its reactivity with cellular components is just balanced by the O_2^- scavenging and repair activities. This balance ensures that the labile dehydratases are almost fully active in air.

Induction of the SoxRS regulon. Upon its discovery, the SoxRS regulon was thought to respond to O_2^-, since the regulon is fully activated by superoxide-forming drugs and is able to induce MnSOD. The inefficiency of induction under anaerobic conditions appeared to support the idea that O_2^- was the direct inducer. However, subsequent work has shown that paraquat can partially activate the SoxRS regulon even anaerobically, where there is no chance of O_2^- formation (58, 59), and oxygen may have enhanced the induction merely by chemically oxidizing the reduced paraquat and ensuring that a sufficient amount was in the oxidized form. Gaudu et al. and Hidalgo et al. elegantly demonstrated that the activation of SoxR occurs when its [2Fe-2S] cluster is oxidized (22, 30). It is clear that a number of low-molecular-weight oxidants, including O_2^-, oxygen, and even redox-cycling drugs themselves (which were used in determining the cluster midpoint potential) (10, 21), are capable of oxidizing the cluster directly when present in high concentrations. The problem is to identify the predominant oxidant in vivo. Our data show that physiological concentrations of O_2^- do not efficiently induce the SoxRS regulon. The modest induction that occurred at high O_2^- concentrations was too slight to be physiologically effective. In contrast, paraquat activated the regulon in a superoxide-independent manner, and the resulting fumarase C activity fully compensated for the inactivation of fumarase A.

The simplest interpretation of our results is that SoxRS exists as a general defense against exogenous redox-cycling drugs rather than against O_2^- per se. Superoxide is a component of the stress imposed by these drugs, and the induction of SOD is an appropriate response, since basal SOD synthesis is inadequate to preserve enzyme activity if O_2^- formation is accelerated. However, these drugs also have superoxide-independent mechanisms of toxicity (3, 33, 62). At least two elements of the SoxRS response—the reduction of outer membrane pores (9) and the activation of the NADPH-producing pentose phosphate pathway—can be more easily rationalized as defenses against exogenous redox-cycling drugs than against O_2^- itself. If so, then it is reasonable that SoxR might sense an effect of the antibiotic other than O_2^- stress. Were SoxR focused on O_2^- levels, the response might fail to be activated in microaerobic conditions or might be short-circuited upon SOD induction. Potential signals include the direct oxidation of SoxR by the drugs and the diminution of SoxR rereduction as the NAD(P)H pool is depleted (18, 44, 48).

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