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Superoxide and the Production of Oxidative DNA Damage

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The conventional model of oxidative DNA damage posits a role for superoxide (O_2^-) as a reductant for iron, which subsequently generates a hydroxyl radical by transferring the electron to H_2O_2. The hydroxyl radical then attacks DNA. Indeed, mutants of *Escherichia coli* that lack superoxide dismutase (SOD) were 10-fold more vulnerable to DNA oxidation by H_2O_2 than were wild-type cells. Even the pace of DNA damage by endogenous oxidants was great enough that the SOD mutants could not tolerate air if enzymes that repair oxidative DNA lesions were inactive. However, DNA oxidation proceeds in SOD-proficient cells without the involvement of O_2^-, as evidenced by the failure of SOD overproduction or anaerobiosis to suppress damage by H_2O_2. Furthermore, the mechanism by which excess O_2^- causes damage was called into question when the hypersensitivity of SOD mutants to DNA damage persisted for at least 20 min after O_2^- had been dispelled through the imposition of anaerobiosis. That behavior contradicted the standard model, which requires that O_2^- be present to rereduce cellular iron during the period of exposure to H_2O_2. Evidently, DNA oxidation is driven by a reductant other than O_2^-, which leaves the mechanism of damage promotion by O_2^- unsettled. One possibility is that, through its well-established ability to leach iron from iron-sulfur clusters, O_2^- increases the amount of free iron that is available to catalyze hydroxyl radical production. Experiments with iron transport mutants confirmed that increases in free-iron concentration have the effect of accelerating DNA oxidation. Thus, O_2^- may be genotoxic only in doses that exceed those found in SOD-proficient cells, and in those limited circumstances it may promote DNA damage by increasing the amount of DNA-bound iron.

The ubiquity of superoxide dismutase (SOD) (44) suggests that superoxide, O_2^-, presents a problem for all aerobic organisms. Superoxide is formed inside cells by the autoxidation of a variety of reduced electron carriers and redox enzymes (29). SOD reduces the intracellular concentration of O_2^- to a level with which the cell can cope. Mutant derivatives of *Escherichia coli* (10), *Streptococcus mutans* (48), *Porphyromonas gingivalis* (49), *Saccharomyces cerevisiae* (9, 56), *Neurospora crassa* (47), *Drosophila melanogaster* (50), and *Caenorhabditis elegans* (34) that lack SOD all exhibit severe phenotypic deficiencies.

The molecular bases of these phenotypes have been elusive. Most biomolecules resist univalent redox reactions and are unreactive with O_2^-, which is a single-electron oxidant or reductant of only moderate strength. One way in which O_2^- is thought to cause toxicity is through its participation in hydroxyl radical production. In 1970, Beauchamp and Fridovich demonstrated the production of a hydroxyl radical, OH·, in vitro from H_2O_2 and O_2^- (3). Subsequent studies revealed that the OH· was generated through the mediation of adventitious iron as follows (42):

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \rightarrow OH\cdot + Fe^{3+} + H_2O \text{ (the Fenton reaction)}$$

The OH· is a powerful oxidant that reacts with other biomolecules, including DNA, at virtually diffusion-limited rates. H_2O_2 and O_2^- appear to be unavoidable by-products of aerobic metabolism, and it is presumed that cells contain a small pool of free iron that is drawn upon for metalloenzyme synthesis. Consequently, for the last 20 years it has been accepted that these reactions may enable endogenous oxidants to continually damage the DNA of aerobic organisms.

The most striking evidence implicating O_2^- in the production of DNA damage in vivo has been obtained with *E. coli* strains lacking cytosolic SOD. These strains are hypermutagenic when grown in air-saturated media, suggesting that an increase in O_2^- leads to an increase in DNA damage (16). They are also 10-fold more sensitive to killing by H_2O_2 than are their wild-type counterparts (10). This killing by H_2O_2 can be blocked by cell-permeable iron chelators, confirming that the killing is mediated by Fenton chemistry (33). Furthermore, the manganese-containing SOD (MnSOD) isozyme and endonuclease IV, an oxidative DNA repair enzyme, are coregulated by the SoxRS regulon (11, 21), which implies that oxidative repair enzymes as well as SOD must be induced to protect the cell from oxidative stress by O_2^-. These in vivo observations seem to support the prevailing hypothesis for the role of O_2^- in the production of oxidative DNA damage.

An unsatisfying aspect of this scheme, however, is that the role envisioned for O_2^- is solely that of transferring electrons to free iron. This is surprising, since other biological reductants, such as thiols (10^{-3} M) and NADH (10^{-4} M), can also fulfill this requirement (32, 51, 58) and they are far more abundant in the cell than is O_2^- (10^{-10} M) (29). It is worth considering that O_2^- may potentiate oxidative DNA damage by some other mechanism. Recent work in a variety of laboratories has demonstrated that O_2^- oxidatively excises iron from a group of dehydratases containing [4Fe-4S] clusters (18, 20, 37, 40). It is possible that by doing so, O_2^- may substantially increase the pool of free iron available to catalyze the production of oxidative DNA damage. This paper presents data consistent with this alternative hypothesis.

MATERIALS AND METHODS

Reagents. Deferoxamine mesylate (desferrioxamine), 2,2-dipyridyl, horse heart cytochrome *c*, hydrogen peroxide (30% [wt/vol]), dimethyl sulfoxide, xanthine, bovine xanthine oxidase, thymine, riboflavin, 6-aminonicotinamide, *o*-ni-

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trophenyl-β-D-galactopyranoside (ONPG), tetracycline, ampicillin, chloramphenicol, nitroblue tetrazolium, and kanamycin were purchased from Sigma Chemical Co., St. Louis, Mo. Iron(II) sulfate heptahydrate and manganese(II) chloride tetrahydrate were purchased from Aldrich Chemical Company, Inc., Milwaukee, Wis. Beef liver catalase and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were purchased from Boehringer Mannheim, Indianapolis, Ind. Coomassie protein assay reagent was purchased from Pierce, Rockford, Ill. Water used in experiments was from the house deionized system and was further purified by a Labconco Water Pro PS system.

Media and cell growth. Cultures were routinely grown in Luria-Bertani (LB) glucose medium containing 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter supplemented with 0.2% glucose. Some cultures were grown in minimal A medium containing minimal A salts (45) supplemented with 1 mM $MgSO_4 \cdot 7H_2O$, 0.2% glucose, 0.5 mM the 20 L-amino acids, and 5 mg of thiamine per liter. When indicated by the strain genotype, 200 μg of thymine per ml was included to support *thyA* mutants. LB plates and minimal A plates were made as described above, with the addition of 15 g of agar per liter. Anaerobic cultures were maintained in a Coy anaerobic chamber under 85% N_2, 10% H_2, and 5% CO_2. Anaerobic media and plates were moved into the anaerobic chamber while still hot and allowed to equilibrate anaerobically at least 1 day prior to use. Plasmids were maintained by antibiotic selection with 50 μg of kanamycin per ml, 20 μg of chloramphenicol per ml, 12 μg of tetracycline per ml, or 100 μg of ampicillin per ml in LB medium. $MnCl_2$ (100 μM) was added to strains containing the MnSOD plasmid to ensure that enough manganese was available to charge the active site.

Optical densities (OD) of cultures were routinely measured at 600 nm. Oxygen consumption of aerobic cultures was measured by using a Clarke oxygen electrode (Rank Brothers, Cambridge, England).

Strain constructions. Strains and plasmids are listed in Table 1. P1 transduction was performed as previously described (45). Transductants receiving mutant *xth*, *recA*, *recB*, or *fnr* genes were selected for the resistance to tetracycline conferred by the linked *zdh-201*::Tn*10*, *srlC300*::Tn*10*, *fuc-3072*::Tn*10*, or *zjc*::Tn*10* allele, respectively. In general, mutant *sodB* and *fur* alleles were selected by resistance to kanamycin; *sodA* and *feo* alleles were selected by resistance to chloramphenicol. The *sodB* allele was transduced by linkage to *zdg-299*::Tn*10* in the construction of AS204, and the *fur* mutation was linked to *zbf-507*::Tn*10* in the construction of KK216. The *thyA* mutant KE110 was selected from JC9239 by resistance to trimethoprim (45). Inheritance of mutations that disrupt recombination was confirmed by screening for UV sensitivity, inheritance of the *xthA-pncA* deletion was confirmed by resistance to 6-aminonicotinamide (57) and inheritance of mutant *sod* alleles was confirmed by the absence of iron-containing SOD (FeSOD) or MnSOD bands on SOD activity gels (4). Potential *fnr* mutants were screened for the inability to grow anaerobically on lactate-nitrate plates (53). Transductants containing the *fur* mutation were identified by their inability to repress an *iucC*::*lacZ* fusion in iron-rich medium. The ColV-K30 plasmid containing *iucC*::*lacZ* was mated into transductants, and the exconjugants were then streaked onto minimal A X-Gal plates containing 40 μM $FeSO_4$. Only exconjugants containing a *fur* mutation and the plasmid form blue colonies. This screen was modified from that of Stojiljkovic et al. (54).

Plasmid manipulations were done by standard protocols (52). Construction of the pKK1 plasmid was accomplished by digesting pHS1-4 (containing *sodB*) and pBR328 with *Pst*I. The *Pst*I fragment of pHS1-4 was then ligated into pBR328 and transformed into AB1157. Transformants were screened for SOD overproduction by using SOD activity gels (4) and SOD solution assays (43). Strains containing the ColV *iucC*::*lacZ* plasmid were constructed by conjugation (45). The exconjugants containing the plasmid formed blue colonies on minimal A plates supplemented with 40 mg of X-Gal per liter and 2 mM 2,2-dipyridyl (54).

Aerobic plating efficiency and viability. To determine the ability of attenuated strains to form colonies in air, cultures were grown anaerobically in LB medium to exponential phase, diluted in anaerobic medium, and then top spread onto anaerobic and aerobic LB plates. Colonies were counted after 24 h. The aerobic plating efficiency was calculated by dividing the number of colonies obtained on aerobic plates by the number on anaerobic plates.

To measure the rate of cell death during exposure to air, exponentially growing anaerobic cultures were diluted approximately 10-fold into prewarmed air-saturated medium. The cultures were incubated with vigorous shaking. At intervals, aliquots of the aerobic cultures were moved back into the anaerobic chamber and plated on anaerobic LB medium. The survival at a given time point is expressed relative to the number of CFU immediately before the exposure to air.

Killing by H_2O_2. Strains were assessed for H_2O_2 sensitivity as previously described (30). To determine the extent of aerobic killing by H_2O_2, cells were grown in LB medium for at least 4 generations to an OD at 600 nm (OD_{600}) of 0.1 to 0.2. Culture densities were adjusted to an OD of 0.1 (3×10^7 CFU/ml), and 1-ml aliquots of cells were challenged with H_2O_2. Challenges were terminated by 625-fold dilution into LB medium. To ensure that the killing of repair-defective strains stopped immediately, 130 U of catalase per ml was included in the diluent. Cells were plated onto LB plates in 2 ml of top agar and incubated 16 to 24 h at 37°C. For anaerobic challenges, cultures were grown to an OD_{600} ranging from 0.025 to 0.1 and adjusted to an OD of 0.025 for H_2O_2 exposure. For those experiments, the H_2O_2 challenge, dilution, and plating were all performed with anaerobic media. Results depicted in the figures and tables are representative of experiments that were repeated a minimum of three times. Although there exists a small experiment-to-experiment variation due to unknown cell growth parameters, in general the log of the surviving fraction of cells varied by less than 20%.

Filamentation. After exposure to H_2O_2, cultures were examined for the presence of filaments (30). Cells were grown to an OD_{600} of 0.2 and challenged for 10 min with 2.5 mM H_2O_2. Killing was stopped by the addition of 1.3×10^4 U of catalase per ml. The cells were then spotted onto LB plates without further dilution and incubated at 37°C. Plates were examined after 2 h by using a Nikon Optiphot microscope at a magnification of ×400.

Enzyme assays. β-Galactosidase activities were determined by a modification of the assay described by Miller (45). Cultures grown to an OD of 0.1 were centrifuged, washed, resuspended in 50 mM Tris buffer (pH 8), and lysed by passage through a French press. Extracts were assayed with ONPG as described above. β-Galactosidase units were defined as the amount of enzyme that produces 1 μmol of *o*-nitrophenol per min at 28°C, pH 7.0, with a millimolar extinction coefficient of 4.5 for *o*-nitrophenol. SOD solution assays were performed by the xanthine oxidase-cytochrome *c* method (43). The total protein content of cell extracts was quantitated with a Coomassie stain-based assay (Pierce).

RESULTS

Superoxide contributes to cell death from oxidative DNA damage. Strains of *E. coli* that lack both cytosolic SODs are approximately 10-fold more sensitive to killing by 2.5 mM H_2O_2 than are their SOD-proficient counterparts (Table 2) (10, 31). We have made several observations that suggest that death is due to DNA damage. First, virtually all of the SOD-deficient cells filamented in the hours after challenge, including the >90% that were scored as dead by their inability to form colonies. The filamentation indicated that the lethal lesion specifically blocked DNA replication, since most other growth processes, such as membrane and protein synthesis, were evidently unhindered.

Second, mutations that diminished the ability of SOD mutants to repair DNA lesions greatly amplified their sensitivity to H_2O_2. Recombination provides an important pathway for the repair of oxidized DNA in *E. coli* (1, 31), and when a *recC sodA sodB* mutant was challenged with exogenous H_2O_2, it was killed extremely rapidly (Fig. 1). Calculations of killing rates indicate that the *recC* mutation acted synergistically with the SOD mutations in conferring the H_2O_2 sensitivity, implying that O_2^- either accelerates DNA damage or disables a complementary DNA repair pathway.

Third, DNA damage by H_2O_2 was previously shown to be mediated by chelator-accessible intracellular iron (33). When cell-permeable iron chelators were added to SOD^- mutants 5 min prior to challenge with H_2O_2, they were largely protected (Table 2). The small amount of persistent killing may have been due to an inability to perfuse sufficient chelator into the cell; higher concentrations of chelator could not be used because of their limited aqueous solubility.

These in vivo results demonstrate, first, that O_2^- contributes to lethality from oxidative DNA damage, at least in SOD-deficient cells. Second, the lethal damage is mediated by the Fenton reaction. These results are in accordance with the standard model of oxidative DNA damage.

Superoxide accelerates oxidative DNA damage. The vulnerability of SOD mutants to H_2O_2 could in principle be due either to an increase in the rate of DNA damage or to disruption of DNA repair processes. The latter possibility was tested by the creation of mutant derivatives that lack both SOD activity and function of the major pathways of oxidative DNA repair. If the major effect of O_2^- were to incapacitate these pathways, then the DNA repair mutations would be epistatic with SOD deficiency.

Genetic studies have indicated that recombinational and excision processes provide alternative mechanisms of repairing the strand breaks that are formed by the oxidation of ribose moieties (14). The elevated sensitivity to H_2O_2 of the SOD^- $RecC^-$ derivative (described above), which lacks the major

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
Strains		
AB1157	F$^-$ *thr-1 leuB6 proA2 his-4 thi-1 argE2 lacY1 galK2 rpsL supE44 ara-14 xyl-15 mtl-1 tsx-33*	31
AS118	As JI130 plus *srlC300*::Tn*10 recA56*	P1(JC10240) × JI130
AS119	As JI131 plus *srlC300*::Tn*10 recA56*	P1(JC10240) × JI131
AS142	As DM49 plus (*sodA*::*Mud PR13*)*25* (*sodB-kan*)*1*-Δ*2*	P1(JI132) × DM49
AS150	As KE110 plus (*sodA*::*Mud PR13*)*25* (*sodB-kan*)*1*-Δ*2*	P1(JI132) × KE110
AS181	As JC7623 plus *fuc-3072*::Tn*10*	P1(JK1015) × JC9239
AS186	As JI132 plus *recB21 recC22* (?) *fuc-3072*::Tn*10*	P1(AS181) × JI132
AS187	As AS150 plus *recB21 recC22* (?) *fuc-3072*::Tn*10*	P1(AS181) × AS150
AS191	As BW9109 plus *nfo-1*::*kan*	P1(BW527) × BW9109
AS196	As BW527 plus (*sodA*::*Mud PR13*)*25* (*sodB-kan*)*1*-Δ*2*	P1(JI132) × BW527
AS204	As AS191 plus (*sodA*::*Mud PR13*)*25* (*sodB-kan*)*1*-Δ*2 zdg-299*::Tn*10*	P1(JI132) × AS191
AS217	As JC9239 plus *recB21 recC22* (?) *fuc-3072*::Tn*10*	P1(AS181) × JC9239
BN407	As AB1157 plus Δ*lacU169* and ColV-K30 (*iucC*::*lacZ*)	2
BW527	As AB1157 plus *nfo-1*::*kan*	13
BW543	As AB1157 plus *xth-1 zdh-201*::Tn*10*	B. Weiss
BW9109	As AB1157 plus Δ(*pncA-xthA*)*90*	B. Weiss
CSH7	*lacY rpsL thi-1*	41
DM49	As AB1157 plus *lexA3*	46
GI69	F$^-$ *aroD shiA proA argE zdg-299*::Tn*10*	22
GW1030	F$^-$ *lac*Δ(*U169*) *thr-1 leu-6 his-4 argE3 galK2 strA31 ilv*(Ts) *tif-1 sfiA11 dinB1*::*Mud* (Ap^r *lac*$^+$)	36
JC6721	*gal endA recC22*	31
JC7623	As AB1157 plus *sbcB15 recB21 recC22*	38
JC9239	As AB1157 plus *recF143*	26
JC10240	HfrPO45 *srlC300*::Tn*10 recA56 thr-30 ilv-318 rpmE300*	12
JI130	As AB1157 plus (*sodA*::*Mud PR13*)*25*	31
JI131	As AB1157 plus (*sodB-kan*)*1*-Δ*2*	31
JI132	As AB1157 plus (*sodA*::*Mud PR13*)*25* (*sodB-kan*)*1*-Δ*2*	31
JI171	As AB1157 plus pDT1-5	This study
JK1015	*fuc-3072*::Tn*10*	D. Clark
JW1071	*valS uxuBA zbf-507*::Tn*10 trp-49*(Am) *lacZ125*(Am) *relA spoT1*	B. Bachmann
KE110	As JC9239 plus *thyA*	This study
KK151	As UM1 plus *srlC300*::Tn*10 recA56*	P1(JC10240) × UM1
KK155	As AB1157 plus *xth-1 zdh-201*::Tn*10*	P1(BW543) × AB1157
KK156	As KK155 plus pDT1-5	This study
KK159	As UM1 plus Δ(*xthA-pncA*) 90 *zdh-201*::Tn*10*	P1(PD12) × UM1
KK165	As UM1 plus (*sodA*::*Mud PR13*)*25* (*sodB-kan*)*1*-Δ*2*	P1(JI132) × UM1
KK170	As AB1157 plus pKK1	This study
KK171	As KK155 plus pKK1	This study
KK181	As AB1157 plus Δ*fnr* (*Bsm-Mlu*)::Ω (Sp^r) *zjc*::Tn*10*	P1(RZ8457) × AB1157
KK195	As JI132 plus ColV-K30 (*iucC*::*lacZ*)	This study
KK197	As JI132 plus pHS1-4	This study
KK201	As KK204 plus ColV-K30 (*iucC*::*lacZ*)	This study
KK203	As AB1157 plus Δ*feoB*::*cat*	P1(QC2130) × AB1157
KK204	As AB1157 plus *fur*::Tn*5*	P1(NC522) × AB1157
KK210	As KK204 plus *zbf-507*::Tn*10*	P1(JW1071) × KK204
KK216	As JI132 plus *fur*::Tn*5 zbf-507*::Tn*10*	P1(KK210) × JI132
KK220	As BW9109 plus Δ*fnr* (*Bsm-Mlu*)::Ω (Sp^r) *zjc*::Tn*10*	P1(RZ8457) × BW9109
KK221	As BW9109 plus Δ*feoB*::*cat*	P1(QC2130) × BW9109
NC522	As GC4468 plus *arcA fur*::Tn*5 fnr-1 zci*::Tn*10*	24
PD12	*argA21 lysA21 malA1 str-104 supE44* Δ(*xthA-pncA*)*90 zdh-201*::Tn*10*	R. Tuveson
QC2130	*thr-1 leuB6 tonA21 lacY1 supE44 rfbD1 thi-1 recD1009* Δ*feoB*::*cat*	D. Touati
RZ8457	Δ*fnr* (*Bsm-Mlu*)::Ω (Sp^r) *zjc*::Tn*10*	P. Kiley
SWC114	As BW9109 plus (*sodA*::*Mud PR13*)*25* (*sodB-kan*)*1*-Δ*2*	P1(JI132) × BW9109
UM1	As CSH7 plus *katE1 katG14*	41
Plasmids		
pKK1	pBR328 with *sodB*	This study
pHS1-4	pBR322 with *sodB*	10
pDT1-5	pBR322 with *sodA*	10

pathway of recombinational DNA repair, indicated that RecBC-driven recombination is functional in SOD mutants. In fact, although *recA56* or Δ*recA* mutants—which are completely defective at DNA recombination—grow well in aerobic medium, the additional absence of SOD activity causes them to die rapidly unless they are maintained in strict anaerobiosis (Fig. 2A). Such strains could be constructed only in an anaerobic chamber. Death was apparently due to Fenton reaction-mediated DNA damage, since the mutants remained alive when 50 μM dipyridyl was added to the aerobic medium.

TABLE 2. Killing of aerobic cells by 2.5 mM H_2O_2

Strain	Relevant genotype	Exposure time (min)	% Survival		SOD (U/mg)
			No addition	+ Dipy[a]	
AB1157	Wild type	10	58	68	7
JI130	*sodA*	10	49	71	2
JI131	*sodB*	10	57	100	3
JI132	*sodA sodB*	10	6.3	66	<0.0
JI171	Wild type (pMnSOD)	10	69	ND[b]	83
JC10240	*recA*	4	5.8	65	ND
KK155	*xth*	4	1.8	75	8
KK156	*xth*(pMnSOD)	4	4.4	ND	125

[a] 1 mM 2,2-dipyridyl was added 5 min prior to challenge with 2.5 mM H_2O_2. Similar protection was obtained with 20 mM deferoxamine.
[b] ND, not done.

Aerobic inviability was also exhibited by *sodA sodB recB recF* mutants, which are SOS proficient but defective at recombination, but not by *sodA sodB lexA3* mutants, which are SOS defective but recombination proficient (Table 3). We conclude that recombinational repair is essential if SOD mutants are to survive in oxygen. Similar results were recently reported by Touati et al. (55). Thus, because O_2^- augmented the oxidant sensitivity of Rec^- strains, it must have a genotoxic effect other than the disruption of recombinational repair.

The known pathway for excision repair of oxidized sugars requires the function of either exonuclease III or endonuclease IV, both of which excise sugar fragments from the 5′ side of the lesion and thereby restore a primer for repair synthesis (15). These enzymes have overlapping specificity, and either one was able to support the aerobic growth of SOD-defective strains. However, when *sodA* and *sodB* mutations were introduced into an *xth nfo* double mutant, the resultant strain struggled to grow in air (Fig. 2B). This strain also formed only 13% as many colonies on aerobic medium as it did on anaerobic medium (Table 3). These extreme synergisms between defects in SOD synthesis and DNA repair confirmed that the genotoxic effect of O_2^- is not to disrupt the function of either of the known repair pathways.

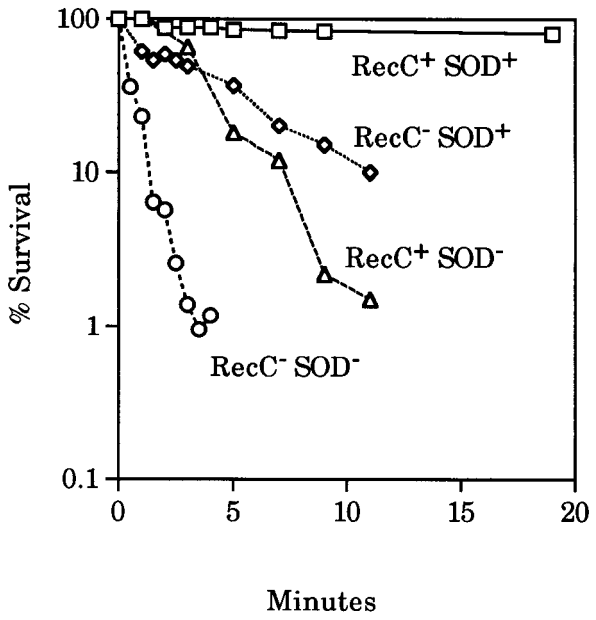

FIG. 1. Superoxide accelerates the rate of DNA damage in a *recC* strain. AB1157 (wild type), JI132 (*sodA sodB*), JC6721 (*recC*), and AS186 (*recC sodA sodB*) were grown aerobically to early log phase in LB medium and challenged with 2.5 mM H_2O_2 as described in Materials and Methods. The relative rates of killing of the SOD^-, Rec^-, and SOD^- Rec^- strains are approximately 10-fold, 5-fold, and 35-fold that of wild-type strains, respectively.

It remained formally possible that O_2^- might inactivate an undiscovered repair pathway that operates independently of RecA, exonuclease III, and endonuclease IV. To test this idea, SOD-deficient and wild-type strains were grown and challenged anaerobically with H_2O_2 and then plated both aerobically and anaerobically. Because O_2^- was not present before or during the challenge, it had no opportunity to affect the rate of DNA damage. However, O_2^- did have the opportunity to disable the repair process of the aerobic outgrowers of the SOD^- mutant, since these cells were exposed to air during the several-hour repair period (31) after the H_2O_2 exposure. The data showed that the survival rates did not vary between SOD-proficient and -deficient cells and that the aerobic and anaerobic plating efficiencies were essentially indistinguishable (data not shown). Collectively, these results confirmed that O_2^- does not disable DNA repair functions but rather that it accelerates the rate of DNA damage.

DNA damage occurs in SOD-proficient cells without the involvement of O_2^-. In the current model of oxidative DNA damage, O_2^- acts as the donor of unpaired electrons to DNA-bound iron. This model has been challenged because of the abundance of other cellular reductants which should be competent to reduce iron. In fact, previously published results suggested that cell killing by H_2O_2 may even occur during anoxia (30). Under such circumstances O_2^- can be presumed to be utterly absent, since intracellular O_2^- arises from the adventitious reduction of molecular oxygen, and so the persistence of DNA damage would prove that O_2^- is not an obligatory participant.

Both wild-type and repair-defective strains were killed during anaerobic exposure to 2.5 mM H_2O_2 (Table 4). The post-challenge filamentation of the non-colony-forming cells, and the hypersensitivity of DNA-repair-defective strains, established that the lethality was due to DNA damage. Exogenous chelators protected against the killing, indicating that the killing was mediated by the Fenton reaction.

During this challenge, trace amounts of oxygen were generated as a by-product of H_2O_2 decomposition by catalase. The amounts produced during the 3-min challenge are very small—less than 1 μM, compared with 210 μM in air-saturated media—but could be a potential source of O_2^-. Therefore, the repair mutations were transduced into strains devoid of catalase. The acute anaerobic sensitivity to H_2O_2 persisted in these strains (Table 4). This was also true when FeSOD was overproduced from a plasmid (Table 4). Thus it is evident that oxidative DNA damage can occur without the mediation of O_2^-. Clearly, there exists a cellular reductant other than O_2^- that is fully capable of driving the DNA damage process.

Although O_2^- does not appear to be required for the production of oxidative DNA damage, it does potentiate DNA damage in mutants devoid of SOD. A critical point is to determine whether O_2^- contributes to damage in SOD-proficient strains as well. Since intracellular concentrations of O_2^- vary inversely with SOD titer, this possibility was tested by seeing whether increases in SOD content above wild-type levels would reduce the amount of damage produced by H_2O_2. Transformation of an MnSOD-overproducing plasmid into SOD-proficient strains permitted a 10- to 15-fold overproduction of SOD but completely failed to protect aerobic cells from

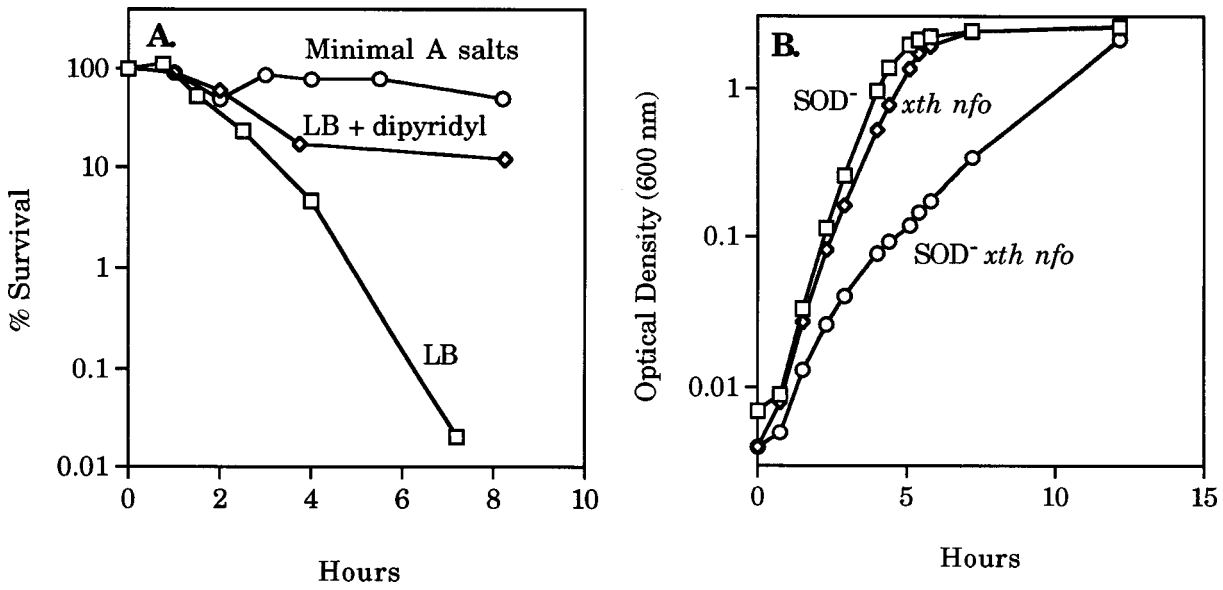

FIG. 2. SOD mutants that lack major repair pathways cannot survive in air. (A) The SOD$^-$ *recA56* strain (KE76) was shifted at time zero from anaerobic to air-saturated medium, and aliquots were removed at intervals and tested for viability (Materials and Methods). Oxidative DNA damage does not occur in minimal A salts lacking a carbon source (30). Dipyridyl at 50 μM was included in the LB medium as indicated. (B) JI132 (SOD$^-$), AS191 (*xth nfo*), and AS204 (SOD$^-$ *xth nfo*) were shifted at time zero from anaerobic to air-saturated LB medium, and outgrowth was monitored by measuring OD_{600}. The aerobic doubling times were 33, 29, and 78 min, respectively.

killing by H_2O_2. This result was especially clear with the repair-defective *xth* strain (Table 2).

Furthermore, single mutations in either *sodA* or *sodB* also did not further sensitize either repair-proficient (Table 2) or RecA-deficient (data not shown) strains to killing by H_2O_2. Evidently, wild-type cells contained more than enough SOD to render insignificant the contribution of O_2^- to DNA damage. A caveat is that this result may not pertain to growth conditions or drugs that might amplify the rate of endogenous O_2^- production.

In SOD-deficient cells the role of O_2^- in genotoxicity is not to deliver electrons to free iron. The above results indicated that O_2^- did not contribute appreciably to DNA damage in SOD-proficient cells. However, the mechanism by which O_2^- promoted damage in SOD$^-$ mutants was unexplained. *E. coli* clearly contains other reductants that can deliver electrons to iron during H_2O_2 exposure. That fact did not, however, rule out the possibility that O_2^- might increase the rate of damage by accelerating this step through its action as an additional reductant. An alternative, suggested in the introduction, is that excess O_2^- might increase the amount of loose iron in the cytoplasm by oxidatively excising it from labile iron-sulfur clusters. That iron would then be available to catalyze the production of DNA lesions. These models can be experimentally distinguished because the former requires that O_2^- be present during the actual period of H_2O_2 exposure in order to continuously rereduce iron atoms as they are oxidized by the H_2O_2. However, if O_2^- increases damage by leaching iron from metalloproteins, and if the cell is slow to restore iron homeostasis, then the sensitivity to H_2O_2 might persist for some time after O_2^- disappears.

SOD-deficient and wild-type strains were grown aerobically

TABLE 3. Aerobic plating efficiencies of SOD$^-$ and repair-defective strains[a]

Repair genotype[b]	Plating efficiency	
	SOD$^+$	SOD$^-$
Repair proficient	1.0	1.2
recA	1.2	3×10^{-7}
recC	1.2	5×10^{-3}
recF	1.2	1.3
recB recF	1.2	1×10^{-4}
lexA	1.4	0.91
xth	1.0	1.3
nfo	1.1	1.1
xth nfo	0.91	0.13

[a] Plating efficiencies were calculated by comparing the number of colonies formed aerobically to the number formed anaerobically (Materials and Methods).
[b] Strains used were AB1157, JI132, JC10240, KE76, JC6721, AS186, JC9239, AS150, AS217, AS187, DM49, AS142, BW9109, SWC114, BW527, AS196, AS191, and AS204.

TABLE 4. Anaerobic killing by 2.5 mM H_2O_2

Strain	Relevant genotype	Exposure time (min)	% Survival	
			No addition	+ Dipy[a]
Expt 1				
CSH7	Wild type	10	57	100
UM1	*katE katG*	10	42	84
KK165	*katE katG sodA sodB*	10	40	70
KK159	*katE katG xth*	3	1.3	40
KK151	*katE katG recA*	3	1.0	48
Expt 2				
AB1157	Wild type	10	17	ND[b]
KK170	Wild type(pFeSOD)	10	14	ND
KK155	*xth*	3	4.9	34
KK171	*xth*(pFeSOD)	3	0.22	ND

[a] 1 mM 2,2-dipyridyl was added 5 min prior to challenge with 2.5 mM H_2O_2. Similar protection was obtained with 20 mM deferoxamine (data not shown).
[b] ND, not done.

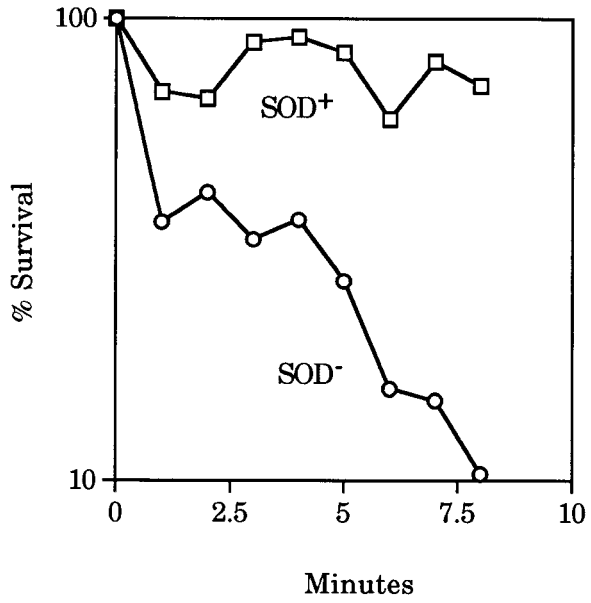

FIG. 3. Sensitivity to H_2O_2 of an SOD^- mutant persists after the disappearance of superoxide. Both UM1 (*katE katG*) and KK165 (*katE katG sodA sodB*) were grown to an OD of 0.2 aerobically in LB medium and then transferred into fully anaerobic medium (Materials and Methods). After an additional 10 min, cells were challenged with 2.5 mM H_2O_2.

to early log phase and abruptly shifted to anaerobiosis. To ensure complete anaerobiosis, cultures were allowed to incubate anaerobically for 10 min to consume >99% of their O_2 supply as measured by a Clarke electrode. The rate of spontaneous dismutation is sufficiently rapid (19) that 90% of the intracellular O_2^- should have disappeared within 10 s of the imposition of anaerobiosis. The cells were then further diluted 1:50 into anaerobic medium and challenged with 2.5 mM H_2O_2. Figure 3 demonstrates that the SOD^- mutant was still approximately 10-fold more sensitive to killing by H_2O_2 than was its SOD-proficient parent. Thus the sensitivity of the SOD^- mutant persisted far beyond the lifetime of O_2^-. Superoxide evidently produced some type of cell damage which, in turn, promoted DNA oxidation even after the O_2^- itself had dissipated.

The amount of intracellular iron determines the rate of DNA damage. To date, the only moiety which O_2^- has been demonstrated to damage directly in vivo is a subclass of [4Fe-4S] clusters (18, 20, 37, 40). Biosynthetic and catabolic pathways that rely upon dehydratases containing such clusters fail to function in SOD mutants, resulting in characteristic phenotypic deficiencies. O_2^- oxidizes these clusters, causing them to disintegrate and spill iron into the cytoplasm. Several lines of evidence predict that the consequent increases in the free-iron concentration will accelerate the rate of oxidative DNA damage.

First, an earlier study determined that prolonged anaerobiosis increases the sensitivity of cells to H_2O_2 (39). The effect was mediated by the induction of a then-unidentified member of the Fnr regulon. That result was repeated (Table 5), and once again the extensive filamentation after the end of the challenge, and the anaerobic sensitization of repair-defective strains, indicated that cell death was due to DNA damage. Kammler et al. have demonstrated that Fnr activates expression of the *feo* gene, which encodes an iron(II) import protein (35), and so we examined the possibility that this was the H_2O_2-sensitizing protein. As shown in Table 5, anaerobiosis

TABLE 5. Anaerobic vulnerability to oxidative DNA damage is due to Feo-mediated iron import

Strain	Relevant genotype	Exposure time (min)	% Survival	
			Aerobic	Anaerobic
AB1157	Wild type	10	75	7.2
KK181	Δ*fnr*	10	95	78
KK203	*feo*	10	75	56
BW9109	Δ*xth*	3	3.2	9.4[a]
KK220	Δ*xth* Δ*fnr*	3	5.9	73
KK221	Δ*xth feo*	3	2.2	54

[a] The anaerobic sensitivity of *xth* mutants is moderated by the absence of molecular oxygen, which in aerobic cells facilitates the decomposition of ribosyl radicals into the strand breaks that are substrates for exonuclease III (28).

did not increase the killing of *feo* mutants. We presume that the induction of Feo during anaerobiosis leads to an increased intracellular iron concentration and a consequent enhancement of the ability of H_2O_2 to damage DNA.

Second, Touati et al. (55) have reported that *fur* mutants, which lack the repressor of iron transport systems (23), are unusually sensitive to H_2O_2. They also found that a *fur recA* mutant was aerobically inviable and from these results concluded that the lethality was due to iron-assisted DNA damage. Those data were reproduced by us (Fig. 4). We observed that in this case the derepressed iron transport system was not Feo, since *fur feo* strains were as sensitive to H_2O_2 as were *fur* Feo^+ strains (data not shown).

Given these results, it seemed possible that O_2^- might promote DNA damage by derepressing the Fur regulon. However, we found that expression of an *iucC*::*lacZ* fusion was unaffected by SOD deficiency (data not shown). Further, the sensitivity to H_2O_2 conferred by deficiencies in SOD and Fur were

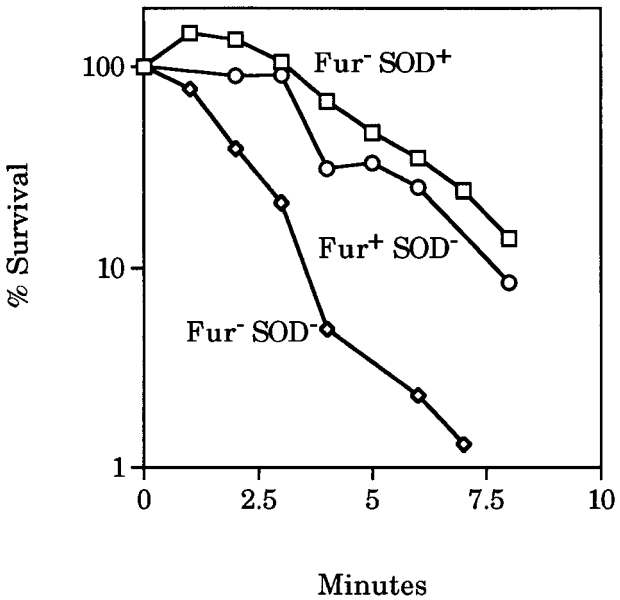

FIG. 4. Mutations in *fur* and SOD independently accelerate DNA damage. JI132 (*sodA sodB*), KK204 (*fur*), and KK216 (*sodA sodB fur*) were grown and challenged with H_2O_2 in amino acid-supplemented minimal A medium as described in Materials and Methods. Relative killing rates, calculated by the amount of time required for 1 log unit of killing, were 1.0 for KK204, 1.1 for JI132, and 2.4 for KK216.

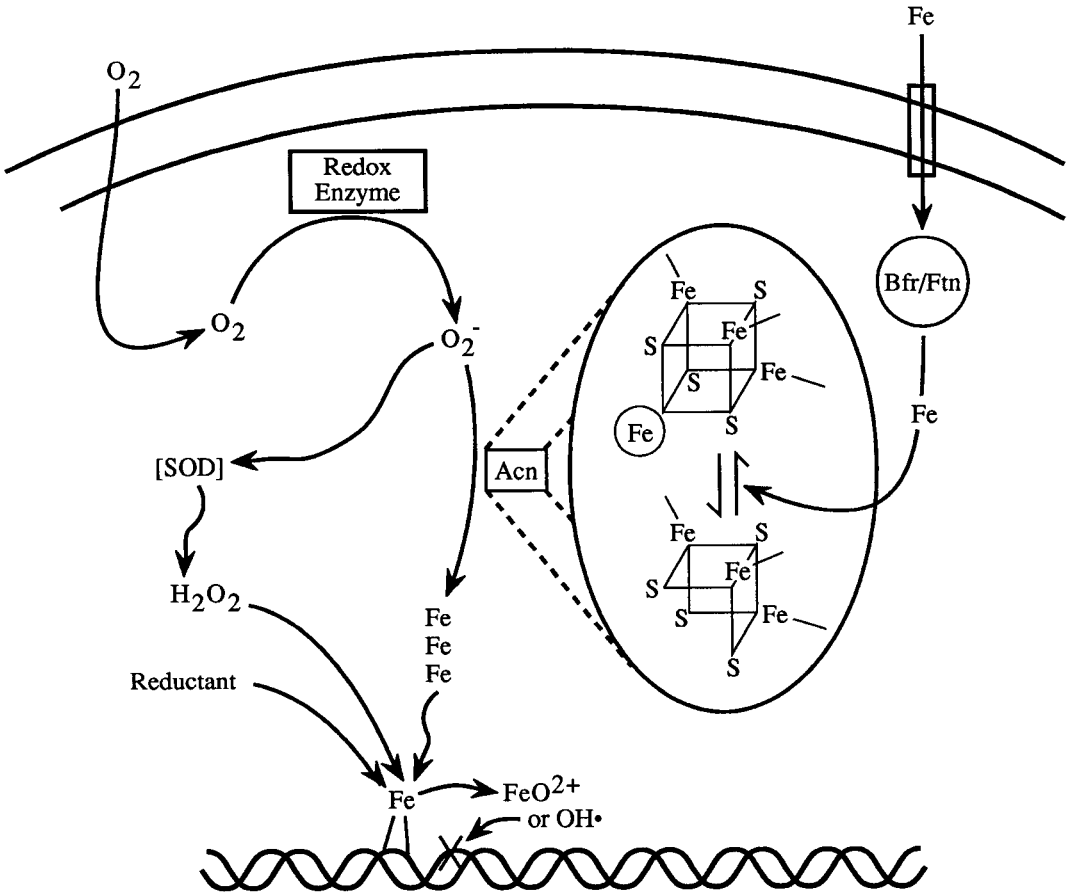

FIG. 5. Model for the role of O_2^- in oxidative DNA damage. Spontaneous autoxidation of respiratory and cytosolic redox enzymes forms O_2^- and, after dismutation, H_2O_2. The O_2^- repeatedly destroys labile [4Fe-4S] clusters (shown here, in aconitase) and thereby causes a steady release of uncoordinated iron into the cytosol. The flux of free iron may be amplified when clusters are recharged with iron drawn from storage reservoirs. A fraction of the free iron will bind DNA and catalyze electron transfer from an unidentified reductant to H_2O_2; the resultant ferryl or hydroxyl radical attacks the adjacent DNA. Abbreviations: Acn, aconitase; Bfr, bacterioferritin; Ftn, ferritin.

approximately additive, confirming their independence (Fig. 4).

Thus, processes that increase the amount of free iron in the cytosol also elevate the rate at which H_2O_2 damages DNA. It follows that the promotion of DNA damage by O_2^- could plausibly result from the iron that it knocks out of labile [4Fe-4S] clusters.

DISCUSSION

The mechanism by which O_2^- promotes DNA damage. Oxidative DNA damage is directly mediated by either hydroxyl or ferryl radicals. The production of these species requires only H_2O_2, accessible iron, and a reductant capable of transferring an electron to iron. In early efforts to explain the toxicity of O_2^-, it was postulated that O_2^- promoted hydroxyl radical formation by acting as a reductant for DNA-bound iron. This role seemed reasonable, both because O_2^- succeeded as an iron reductant in vitro (42) and because SOD mutants exhibited the hypermutagenicity expected of a strain in which HO· production was rapid (16). However, subsequent measurements of the rate constant for iron reduction by O_2^- (10^5 M^{-1} s^{-1} for Fe^{3+}-ATP) (6) and estimates of the O_2^- content of wild-type cells (ca. 2×10^{-10} M) (29) imply that the half time for iron reduction by O_2^- in vivo would be 10 h, which is far too long to support the rate of DNA damage observed to occur in cells perfused with H_2O_2. The present demonstration that iron mediates oxidative DNA damage even when O_2^- is utterly absent confirms that some other cellular reductant acts as the critical electron donor. In fact, researchers from several laboratories have noted that more-abundant reductants succeed in driving the Fenton reaction in vitro (32, 51, 58). Among these is NADH, and the extreme sensitivity to H_2O_2 of NADH dehydrogenase mutants (32) supports the possibility that NADH fulfills this role in vivo.

Yet O_2^- nevertheless does something to increase the rate of DNA damage. Since this effect is apparent when H_2O_2 is supplied exogenously, O_2^- must elevate the intracellular concentration of one of the other principals, either the unidentified reductant or the catalytic free iron. If the reductant is indeed NADH, the former possibility appears particularly unlikely, because O_2^- would be more likely to diminish than elevate NADH levels, given its ability to inactivate enzymes belonging to the tricarboxylic acid cycle. However, there exist two mechanistically sound routes by which O_2^- may increase the cytosolic concentration of free iron. First, O_2^- leaches iron from the iron-sulfur clusters of a subset of dehydratases. Two of the vulnerable enzymes—aconitase and fumarase—are relatively abundant and have sufficiently high rate constants for inactivation by O_2^- (10^6 to 10^7 M^{-1} s^{-1}) that the flux of iron

into the cytosol could be quite large (18, 25). Furthermore, there exist as-yet-undefined mechanisms that replace the lost iron, perhaps with iron derived from cellular stores (17, 37). This raises the possibility that when O_2^- is abundant the labile clusters might be rebuilt and redestroyed many times apiece, permitting each in effect to catalyze the spillage of multiple iron atoms from storage reservoirs into the cytosol. If the spilled iron were only slowly recovered, then a substantial amount of free iron might accumulate (Fig. 5).

An alternative is that O_2^- liberates iron directly from one or both of the bacterial iron storage proteins, ferritin and bacterioferritin. These proteins are homologous to mammalian ferritins (27, 59), from which O_2^- can reductively leach iron (7, 8). The lability of the bacterial proteins has not yet been tested.

Unfortunately, the amount of free iron present in either normal or superoxide-stressed *E. coli* is unknown; the fact that the vast majority of intracellular iron is present in metalloproteins makes such measurements technically difficult. However, the hypothesis is particularly attractive because the data reported in this study confirm that an increase in intracellular iron levels would indeed have the effect of accelerating DNA damage.

In that regard, it is interesting that iron concentrations may be kept significantly lower in aerobic than in anaerobic cells. This was previously suggested by the observation that apoenzymes appear to be much more readily charged with iron during anaerobiosis (5). This study has indicated that the anaerobic action of the Feo transport system leads to high iron levels that greatly increase the vulnerability to H_2O_2. Perhaps the need to minimize oxidative DNA damage favored the evolution of a particularly stringent control of free-iron content during aerobiosis.

The dosimetry of O_2^- and the generation of oxidative DNA damage. In the experiments reported here, the ability of O_2^- to promote DNA damage was only apparent in mutant cells devoid of SOD. It is uncertain whether cells that have wild-type levels of SOD ever accumulate enough O_2^- to have an impact upon the rate of mutagenesis. It remains possible that the intracellular O_2^- concentration approaches genotoxic levels under conditions in which enzymes that produce O_2^-—including fumarate reductase and NADH dehydrogenase II—are amply synthesized. O_2^- production can also increase manyfold when cells are infused with redox-cycling drugs. However, the notion that O_2^- promotes spontaneous oxidative mutagenesis in the nonattenuated cell should perhaps for now be regarded with caution.

The inviability in air of strains that had been stripped of O_2^- scavengers and of oxidative DNA repair systems underscores the potential hazard to DNA imposed by oxygen. During aeration, these strains succumbed not to H_2O_2 artificially imposed by the experimenter but to endogenous oxidants that arose spontaneously as inadvertent by-products of aerobic metabolism. The synthesis of such defensive systems is apparently prerequisite for survival in aerobic habitats, and the failure of certain microbes to devote resources to this cause must, at least in part, have consigned them to obligate anaerobiosis. It remains to be seen whether enough intracellular oxidants elude the defensive systems of aerotolerant organisms that oxidative lesions comprise a major cause of "spontaneous" mutagenesis and cell death.

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