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Reduced Flavins Promote Oxidative DNA Damage in Non-respiring *Escherichia coli* by Delivering Electrons to Intracellular Free Iron*

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When cells are exposed to external H_2O_2, the H_2O_2 rapidly diffuses inside and oxidizes ferrous iron, thereby forming hydroxyl radicals that damage DNA. Thus the process of oxidative DNA damage requires only H_2O_2, free iron, and an as-yet unidentified electron donor that reduces ferric iron to the ferrous state. Previous work showed that H_2O_2 kills *Escherichia coli* especially rapidly when respiration is inhibited either by cyanide or by genetic defects in respiratory enzymes. In this study we established that these respiratory blocks accelerate the rate of DNA damage. The respiratory blocks did not substantially affect the amounts of intracellular free iron or H_2O_2, indicating that that they accelerated damage because they increased the availability of the electron donor. The goal of this work was to identify that donor. As expected, the respiratory inhibitors caused a large increase in the amount of intracellular NADH. However, NADH itself was a poor reductant of free iron *in vitro*. This suggests that in non-respiring cells electrons are transferred from NADH to another carrier that directly reduces the iron. Genetic manipulations of the amounts of intracellular glutathione, NADPH, α-ketoacids, ferredoxin, and thioredoxin indicated that none of these was the direct electron donor. However, cells were protected from cyanide-stimulated DNA damage if they lacked flavin reductase, an enzyme that transfers electrons from NADH to free FAD. The K_m value of this enzyme for NADH is much higher than the usual intracellular NADH concentration, which explains why its flux increased when NADH levels rose during respiratory inhibition. Flavins that were reduced by purified flavin reductase rapidly transferred electrons to free iron and drove a DNA-damaging Fenton system *in vitro*. Thus the rate of oxidative DNA damage can be limited by the rate at which electron donors reduce free iron, and reduced flavins become the predominant donors in *E. coli* when respiration is blocked. It remains unclear whether flavins or other reductants drive Fenton chemistry in respiring cells.

The appearance of oxygen in the atmosphere allowed some organisms to develop more efficient metabolic schemes, but it also raised the possibility that inadvertent chemical oxidations could disrupt metabolism and damage biomolecules. Molecular oxygen is a triplet species that is constrained to accept electrons one at a time, and its univalent redox potential is low enough that it is unable to abstract electrons from most structural biomolecules. Thus it does not directly oxidize amino acids or nucleic acids. However, it can slowly oxidize the reduced flavins of many redox enzymes, thereby generating a mixture of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (1–3). O_2^- and H_2O_2 are both stronger oxidants than oxygen, and they can efficiently oxidize iron-sulfur clusters and protein thiols with which oxygen reacts slowly or not at all (4, 5) (Reaction 1).

$$O_2 \xrightarrow{-0.33\ V} O_2^- \xrightarrow{+0.94\ V} H_2O_2 \xrightarrow{+0.38\ V} OH^- + HO^\cdot \xrightarrow{+2.33\ V} H_2O$$

REACTION 1

When O_2^- and H_2O_2 are abundant, the resultant damage compromises the function of key enzymes and thereby inhibits metabolism (6, 7).

Still, neither O_2^- nor H_2O_2 is a sufficiently potent oxidant to abstract electrons from nucleic acids (8, 9). Oxidative DNA damage is therefore ascribed to a fourth oxygen species, the hydroxyl radical ($HO^\cdot$). The hydroxyl radical is powerful enough to react at diffusion-limited rates with either the base or sugar residues of DNA, leading to base modification and/or strand breakage (Reaction 4, below) (10, 11). $HO^\cdot$ can be formed when H_2O_2 is univalently reduced by transition metals. In biological systems, the pertinent metal appears to be ferrous iron. The evidence for this conclusion is that cell-permeable iron chelators fully protect intracellular DNA from exogenous H_2O_2 (12, 13). Furthermore, mutants that are derepressed for iron uptake, and as a consequence contain abnormally high levels of free iron, exhibit high mutation rates and are rapidly killed when they are exposed to H_2O_2 (14–16).

Thus the process of DNA damage requires three steps as shown in Reactions 2–4:

$$\text{reductant} + Fe^{3+} \rightarrow Fe^{2+} + \text{oxidized reductant}$$

REACTION 2

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^\cdot$$

REACTION 3, **Fenton reaction.**

$$HO^\cdot + DNA \rightarrow H_2O + \text{lesion}$$

REACTION 4

It is the first step (Reaction 2) that remains unclear. The catalytic iron is thought to exist as a small pool of adventitious iron that has dissociated from iron trafficking processes or metalloenzymes. This iron is presumably loosely bound to whatever biomolecules are available, including DNA itself. (Because it is not stably integrated into proteins, this iron is typically referred to as "free" iron.) Because most ferrous chelates are rapidly oxidized by molecular oxygen, the existence of a small pool of ferrous iron that is available to react with H_2O_2

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TABLE I
Strains and plasmids used in this study

Strain or plasmid	Genotype	Source
AN387	*F⁻ rpsL gal*	66
AN384	As AN387 plus *ubiA420 menA401*	66
ALN2	As AN387 plus *fre*::*kan*	P1(LS1212) × AN387
ALN3	As ALN2 plus *recA938*::*cat*	P1(SM5) × AN387
ALN10	As AN387 plus *zeb-1*::Tn*10* Δ*(edd-zwf)22*	Lab collection
ALN11	As AN387 plus *zeb-1*::Tn*10 pnt*::Tn*5 (edd-zwf)22*	P1(RH5) × ALN10
ALN18	As ALN2 plus *ndh*::*cam*	P1(SLC22) × ALN2
ALN20	As ALN18 plus *cxo*::Tn*10*	P1(JI301) × ALN18
ALN30	As JI367 + pFN3	Lab collection
ALN33	As ALN2 plus *polA12*(ts)	P1(CY375) × ALN2
JI301	As AN387 plus *cxo*::Tn*10 F⁻rpsL gal*	Lab collection
JI367	*katE12*::Tn*10* Δ(*katG17*::Tn10)1	Lab collection
AS457	As AN387 plus *recA938*::*cat*	Lab collection
AS471	As AN387 plus Δ(*xth-pncA*) *zdh201*::*Tn10 rpsL gal*	Lab collection
AB1157	F⁻ *thr-1 leuB6 his-4 thi-1 argE2 lacY1 rspL supE44 ara-14 xyl- 15 mtl-1 tsx-33 galK2*	41
JTG10	As AB1157 plus *gshA20*::*kan*	37
SLC22	As AN387 plus *ndh*::*cam*	Lab collection
KK210	As AB1157 plus *fur*::*kan zbf-507*::Tn*10*	40
DC1186	*pta zfa*::Tn*10 pfl-1 thr-1 leu-6 tonA21 supE44 rpsL thi lacY1*	D. Clark
LS1212	As K12 plus *fre*::*kan*	M. Fontecave
CY375	*lacZ53*(Am) *thyA36 IN(rrnD-rrnE)1 rpsL151 polA12(ts) rha-5 deoC2 fadAB*::*Tn10*	J. Cronan
RH5	As K12 plus *pnt*::*Tn5*	41
Plasmids		
pFN3	pJF119EH derivative containing *fre*	61
pACYC184	Plasmid for nicking assays	A. Salyers
pES1	*fre* overproducer	28

indicates that some countervailing reductive process must continuously re-reduce the free iron. In fact, the efficiency with which exogenous H_2O_2 damages DNA provides evidence that iron re-reduction must be rapid. When *Escherichia coli* is treated with 2.5 mM H_2O_2, DNA damage is generated at a high rate for up to 20 min (13). At this concentration of H_2O_2 the Fenton reaction (76 M^{-1} s^{-1} (17)) should oxidize pre-existing reduced iron within seconds; the observation that damage occurs steadily indicates that a reductant recycles the iron very efficiently.

Thus hydroxyl radicals are created when iron catalyzes the transfer of an electron from a reductant to H_2O_2. That reductant has not been identified. Superoxide itself is capable of reducing free iron and functions in that capacity in some *in vitro* Fenton systems (8, 9, 18). However, the intracellular concentration of superoxide is so low ($\sim10^{-10}$ M) (19, 20), and its reduction of bound iron so slow ($\sim10^{5}$ M^{-1} s^{-1}) (21), that the predicted half-time for this reaction *in vivo* is >10 h, far too long to be physiologically relevant. Although superoxide is mutagenic (22), this effect apparently stems from its ability to increase the concentration of free iron by leaching it from unstable iron-sulfur clusters (16, 23). Other cellular reductants have been considered. Thiols ($\sim10^{-3}$ M), α-ketoacids ($\sim10^{-3}$ M), and NAD(P)H ($\sim10^{-4}$ M) are all abundant inside cells, and each of these can reduce ferric iron *in vitro* (24–26). The rates at which they do so have not been compared, so it is difficult to predict which would be the predominant reductant *in vivo*.

E. coli becomes fully resistant to oxidative DNA damage when it is starved for carbon sources, and sensitivity returns when they are re-administered (27). This pattern matches the depletion and replenishment of these reductant pools, and it suggests that the availability of a reductant for free iron can determine the rate at which oxidative DNA damage occurs. In fact, when respiration in *E. coli* was blocked by the addition of cyanide, the rate at which exogenous H_2O_2 killed cells was dramatically potentiated (26). Such a result would be expected if NADH, or another electron carrier in equilibrium with it, could efficiently reduce free iron. The purpose of this work was to further explore this phenomenon in order to identify that reductant.

MATERIALS AND METHODS

Chemicals—Ferric chloride, ferrous sulfate heptahydrate, potassium cyanide (KCN), magnesium sulfate heptahydrate, and magnesium chloride were purchased from Aldrich. Diethylenetriaminepentaacetic acid, deferoxamine mesylate (desferrioxamine), isopropyl-β-D-thiogalactopyranoside (IPTG),[1] NADPH, NADH, EDTA, kanamycin, ampicillin, hydrogen peroxide (H_2O_2) (30% w/v), tetracyline, reduced glutathione (GSH), chloramphenicol, *Vibrio fisheri* luciferase, isopropyl alcohol, methanol, decanol, methanesulfonic acid methyl ester, L-glutamic dehydrogenase, FAD, deamino-NADH, 5,5′-dithio-bis(2-nitrobenzoic acid), ADP, uracil, and 3-(2-pyridyl)-5,6-bis(2[5-furylsulfonic acid])-1,2,4-triazine (ferene) were purchased from Sigma. Beef liver catalase was from Roche Molecular Biochemicals. Expand Long Template PCR kit was purchased from Roche Molecular Biochemicals. dNTPs were from Promega, and PCR primers were from Operon. Total protein was measured with the Coomassie protein reagent (Pierce). β-Mercaptoethanol and sodium citrate were from Fisher. Water was purified with a Labconco Water Pro system.

Flavin reductase was overproduced and purified as described (28). The purified fraction exhibited a single band at the expected molecular weight, by SDS-PAGE analysis. The enzyme was stored at −80 °C in 20 mM KP_i, pH 7.0, 150 mM NaCl, and 30% glycerol.

Strains and Growth Media—All strains used in this study were K-12 derivatives (Table I). Mutations were moved into strains by P1 transduction (29). Null mutations in *recA*, *xth*, *cyo*, *cyd*, *ndh*, and *nuo* can, in some backgrounds, cause defective growth in aerobic media. Therefore, to avoid the possible accumulation of suppressor mutations during the outgrowth of mutant colonies, these alleles were transduced anaerobically. Other strains were generated in air. Mutations in *xthA* were confirmed by sensitivity to H_2O_2 (30); in *recA*, by sensitivity to ultraviolet radiation; and in *polA*, by inability to grow on 0.01% methanesulfonic acid methyl ester LB plates. *ndh* mutations were screened by the inability of inverted membrane vesicles to oxidize NADH, after incubation at high pH to eliminate *Ndh*I activity (2), and *nuo* mutations by the inability of their vesicles to oxidize deamino-NADH (2). Mutations in

[1] The abbreviations used are: IPTG, isopropyl-β-D-thiogalactopyranoside; pmf, protonmotive force.

zwf, *pnt*, and *fre* were screened by measurement of glucose-6-phosphate dehydrogenase (31), transhydrogenase (32), and flavin reductase activities (33). *fre* gene disruptions were confirmed by PCR analysis.

Defined media contained either M9 or minimal A salts (29), 0.2% glucose, 0.2% acid-hydrolyzed casamino acids, 0.5 mM tryptophan, 1 mM $MgSO_4 \cdot 7H_2O$, and 5 mg of thiamine per liter. LB broth contained 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter supplemented with 0.2% glucose. Anaerobic experiments were conducted in a Coy chamber under 85% N_2, 10% H_2, and 5% CO_2. Media and plates were made anaerobic by moving the samples into the anaerobic chamber while still hot and allowing them to equilibrate with the anaerobic atmosphere for at least 24 h prior to use. Plasmids and strains were maintained by an antibiotic selection with 50 μg/ml kanamycin, 12 μg/ml tetracycline, or 100 μg/ml ampicillin. Where indicated, 150 μg/ml chloramphenicol was used to inhibit protein synthesis. Genes under control of the P*tac* promoter were induced with 50 μM IPTG.

The absorbances of cultures were monitored at 600 nm. Rates of oxygen consumption by aerobic cultures were measured using a Clarke-type oxygen electrode (Rank Brothers, Cambridge, UK).

Killing by H_2O_2 and Ultraviolet Radiation—The rate at which 2.5 mM H_2O_2 killed cells was measured as described previously (27). Briefly, overnight cultures were diluted 1:1000 into fresh media and grown for at least four generations to an A_{600} of about 0.1–0.2. Immediately prior to a H_2O_2 challenge, cells were diluted to an A of ~0.1 in 37 °C medium, and H_2O_2 was added. At timed intervals aliquots were removed, diluted at least 1:100-fold into medium containing 130 units/ml catalase, plated onto LB glucose plates in top agar, and incubated overnight at 37 °C. Where indicated, 3 mM KCN was added 5 min prior to the addition of H_2O_2. For anaerobic challenges, overnight cultures were grown to mid-exponential phase and then diluted to an absorbance of 0.050 for H_2O_2 exposure. In these cases all media, diluent, and reagents were anaerobic, and plating was performed anaerobically. Where indicated, the filamentation of non-colony-forming cells was monitored by microscopy (34).

The sensitivity of cells to ultraviolet radiation was measured using exponentially growing cultures. Cells were placed into a sterile Petri dish and illuminated with a far-ultraviolet lamp. At intervals aliquots were removed, diluted into LB medium, plated in top agar, and incubated at 37 °C overnight. Survival was expressed as a percentage of the initial population.

Measurement of Intracellular Free Iron—Intracellular free iron was quantified by EPR spectroscopy (16). Exponentially growing cells (1.5 liters, A_{600} = 0.1–0.2) in M9 medium were centrifuged at 7800 × *g* at room temperature for 7 min and resuspended in 0.7% of the original volume of the same medium containing 20 mM desferrioxamine. Where indicated 3 mM KCN was added simultaneously with the desferrioxamine. The sample was incubated at 37 °C with shaking for 10 min, centrifuged, washed with cold 20 mM Tris-HCl, pH 7.4, and resuspended in 200 μl of the same buffer containing 10% glycerol. The suspension was loaded into EPR tubes, frozen in a dry-ice bath, and scanned with a Varian Century E-112 X-band spectrophotometer equipped with a Varian TE102 cavity and a Varian temperature controller. The spectrometer settings used were as follows: field center, 1570 G; receiver gain, 2,500; field sweep, 400 G; modulation amplitude, 1.25 G; *T*, −125 °C; and power, 30 milliwatts. To determine the ability of H_2O_2 to oxidize intracellular free iron, 2.5 mM H_2O_2 was added following the initial resuspension of cells, and desferrioxamine was omitted.

Measurement of Polymerase-blocking DNA Lesions—DNA damage can be quantified by the ability of DNA lesions to block PCR amplification of the DNA template (35). Cells were grown to A_{600} = 0.1–0.2, and they were then treated with H_2O_2 and/or KCN as described above. When the *polA*(Ts) strain was studied, cultures were shifted to 42 °C from 30 °C 30 min before the challenge. Catalase (26,000 units/ml) was added to scavenge the H_2O_2. The high amount was necessary to compensate for its substantial inhibition by cyanide. Total DNA was then extracted and purified according to procedures given for the DNeasy DNA extraction kit (Qiagen). DNA was quantitated using the Picogreen system (Molecular Probes).

The DNA template (25–100 ng) was amplified in a 50-μl reaction that contained 0.3 μM primers, 350 μM dNTPs (Promega), 0.2 μCi of [α-^{32}P]dATP, and 2.5 units of the *Taq* polymerase-based Expand Long Template PCR system (Roche Molecular Biochemicals). A 10-kb fragment of genomic DNA near the *fumC* gene was chosen as the target sequence in our experiments. The primers were 5′ → 3′ C-terminal CAGGGCAACGGAACGGAACACCCGCCCAGAGCATAACC and 5′ → 3′ N-terminal CGGCGTGAACTCGCAAAATATTAACGATTCAGC. Amplification was accomplished by 30 cycles of denaturation (30 s, 94 °C), annealing (30 s, 62 °C), and extension (10 min, 65 °C). Control experiments established that the amount of PCR product was proportional to the amount of template DNA over the range of template DNA used in these experiments. The PCR product was resolved on a 1% agarose gel. The dried gels were exposed to a phosphor screen, and the radioactive product was quantified using the ImageQuant PhosphorImager program (Amersham Biosciences).

Measurement of Intracellular NADH and NADPH Pools—The assays of NADH and NADPH were modified from previous procedures (19). In this study, in order to measure levels with sufficient precision, cells were concentrated prior to lysis. Oxygen was bubbled through the cell suspension in order to ensure that the dense culture did not become anaerobic.

Cells were grown in minimal A medium containing 0.2% glucose and 0.2% casamino acids, supplemented with 0.5 mM tryptophan, to A_{600} = 0.100–0.200. Cells were centrifuged for 3 min at room temperature, and the pellet was resuspended at 37 °C in the same medium containing 1% glucose at A_{600} = 1.5–2.0. The cell suspension was bubbled with pure oxygen for 5 min to restore aerobic metabolism, and a sample was then removed to determine the absorbance and number of viable cells. Where appropriate, 3 mM KCN was added for 3 min. In some cases, 2 mM H_2O_2 was added for 3 min. The H_2O_2 was then scavenged with 3500 units/ml catalase for 7 min. Cells were then lysed instantly with 0.125 M NaOH (final pH = 12.0). Alkaline extracts were incubated at 50 °C for 9 min in order to ensure the inactivation of enzymes and then neutralized with HCl to pH 7.6. Debris was removed by centrifugation at 4 °C (20,000 × *g* for 20 min). The supernatants were decanted into separate clean vials, and 2.0 mg/ml oxidized glutathione, 1.0 mg/ml chicken egg albumin, and 1.0 mM EDTA were added. The total reduced pyridine dinucleotide pool (NADH + NADPH) of each sample was measured directly in a luciferase reaction (below). NADH alone was measured after the sample was incubated with 40 units/ml glutathione reductase to oxidize the NADPH. NADPH alone was measured after the addition of 0.5 units/ml of *E. coli* inverted respiratory vesicles to oxidize the NADH. Both glutathione reductase and membranes were added to some samples, to oxidize both NADPH and NADH, thereby providing a depleted extract from which standard curves could be generated upon the addition of defined amounts of NAD(P)H. After 8–10 min incubation of samples with these enzymes at room temperature, the glutathione reductase and/or respiratory membranes were inactivated by the addition of 6 M NaOH to pH 12.0. The pH was then re-adjusted to 7.2.

NAD(P)H levels were determined by the addition of 800 μl of sample to 100 μl of reaction mixture (composed of 10 mM KP_i, 0.05% β-mercaptoethanol, 0.1 mg/ml decanal, and 0.2 mg/ml FMN, at pH 7.0) and 200 μl of luciferase (4.5 mg/ml in water) in a Turner TD-20e luminometer. NADH and NADPH concentrations were calculated from the integrated light emission. Stock solutions of NADH and NADPH that were used to establish standard curves were themselves calibrated by absorbance at 340 nm (ϵ = 6.22 mM^{-1} cm^{-1}).

To test the stability of NADH and NADPH during sample preparation, known amounts were added to initial cell lysates in control experiments. The ultimate recovery was >90%. Cell viability was checked before and after H_2O_2 exposure, and the synergistic toxicity of H_2O_2 and cyanide described in other experiments was reproduced.

Peroxidase Activity—To test whether H_2O_2 can act as a respiratory electron acceptor, inverted membrane vesicles were prepared from aerobic cultures (19). All reagents were made anaerobic, including inverted membrane vesicles, which were flushed with pure N_2 for 5 min prior to being placed in the anaerobic chamber. Anaerobic reactions contained membrane vesicles (0.01 units of NADH oxidase activity), 100 μM NADH, and 2.5 mM H_2O_2 in 50 mM KP_i, pH 7.4. Peroxidase activity was measured by following the oxidation of NADH by H_2O_2 at 340 nm in sealed cuvettes.

Glutathione Measurements—Reduced glutathione (GSH) levels were inferred from measurements of total acid-soluble thiols (36), because >90% of this pool is GSH (37). Cells were grown in LB to A_{600} = 0.2–0.3 and, where indicated, exposed to 3 mM KCN for 5 min. Cultures were then centrifuged, washed in the same medium, and resuspended in 3% of the original volume of cold 50 mM KP_i, pH 7.4, 1 mM EDTA. The sample was lysed by French press and clarified by centrifugation. An aliquot was removed for protein determination. Trichloroacetic acid (2.5%) was added to the remaining sample, and the sample was chilled on ice for 10 min. The sample was clarified by centrifugation and assayed. One-ml reactions consisted of 0.06 mg/ml 5,5′-dithiobis(2-nitrobenzoic acid) dissolved in 50 mM KP_i, pH 7.4, and 100 μl of sample. Final absorbance was determined at 412 nm. A standard curve was generated by spiking the sample with known quantities of GSH.

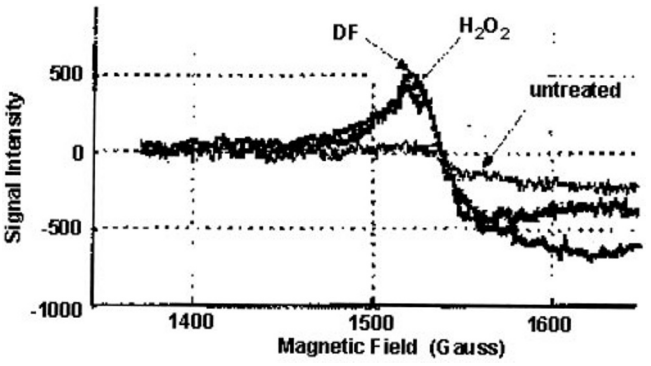

FIG. 1. **EPR analysis of the redox state of intracellular free iron.** Aerobic KK210 (*fur*) cells were grown and prepared as specified under "Materials and Methods." Where indicated, cells were treated with 2.5 mM H_2O_2 or 20 mM desferrioxamine prior to freezing. Data from *fur* mutants are shown because the greater free-iron concentration in these cells (80 μM) (16) enhanced resolution. Wild-type cells exhibited similar responses. The *tracings* shown represent the average of at least 20 scans.

Phage Inactivation—The ability of an *in vitro* system to generate DNA damage was assessed by the inactivation of bacteriophage λ (13). Typically, phage were diluted to 10^5 plaque-forming units/ml into a 0.8% saline reaction mixture at room temperature containing 1–10 μM ferric chloride, 100 μM reductant, and 250 μM H_2O_2. At intervals aliquots were diluted into saline and infected into wild-type *E. coli*, which were plated in top agar. After overnight growth, the number of plaques were counted. The fraction of surviving phage was calculated relative to control phage that were not exposed to the Fenton system.

In Vitro Measurements of the Reduction of Ferric Iron—The rate at which various reductants converted ferric to ferrous iron *in vitro* was measured with a ferene dye-based assay. Reactions were performed at room temperature. They contained 10–20 μM ferric chloride, NAD(P)H, or glutathione, and, where indicated, 15 μM FAD and purified flavin reductase in a final volume of 1.5 ml of 20 mM Tris, pH 7.4. All buffers and reagents were anaerobic.

In reactions that measured iron reduction by crude extracts, reactions were conducted in air, and buffers were not added, because they promote the oxidation of Fe^{2+} by molecular oxygen. Approximately 3 mM KP_i was carried over into the reaction system from the cell extracts. Because the concentration of ferric chloride was low, the pH of this system was about 6.0; it did not change significantly over the course of the reaction.

At time points, 60 μl of a freshly made mixture of 100 mM ADP and 30 mM ferene were added to stop the iron-reduction reactions and chelate the ferrous iron. Ferrous iron was quantified at 562 nm using an extinction coefficient of 26.6 mM^{-1} cm^{-1}.

Generation of DNA Strand Breaks in Vitro—Plasmid pACYC184 DNA was prepared by Qiagen column; the final elution was performed using anaerobic 10 mM Tris, pH 8.0, and the sample was frozen. DNA damage reactions were performed in an anaerobic chamber with reagents that had been degassed or freshly dissolved in anaerobic water. Reactions contained 200 μM NADH, 15 μM FAD, 10 μM $FeCl_3$, Fre, 10 ng of DNA, and 200 μM H_2O_2, added in that order. In some cases 3 mM glutathione, 20 mM desferrioxamine, or 5000 units of catalase were included. Reactions were terminated by the addition of 5000 units of catalase. Samples were then electrophoresed on a 1% agarose gel, stained for 2 h with ethidium bromide, destained for 4 h, and scanned by a PhosphorImager to quantify the bands of supercoiled and relaxed DNA.

RESULTS

Most Intracellular "Free Iron" Is in the Ferrous Form—The vulnerability of *E. coli* to Fenton chemistry suggests that at least some of its free iron is in the ferrous form. Because ferric iron has a sharp and clear EPR signal, whereas that of ferrous iron is broad and indistinct (38), we used EPR spectroscopy to determine the predominant redox state of the free iron. The EPR signal of untreated cells was small, consistent with the spectrum of ferrous iron (Fig. 1). However, the signal could be enlarged and sharpened by incubating cells with desferrioxamine, a cell-permeable iron chelator that binds free iron and stimulates its autoxidation to the ferric form (Fig. 1). (Desferrioxamine chelates iron that is loosely bound to biomolecules but does not remove iron from metalloproteins (16).) A very similar transition occurred when desferrioxamine was omitted and cells were treated instead with high concentrations of H_2O_2. The similarity of the EPR signals implied that most of the iron that could be visualized in the desferrioxamine-treated cells had been in the ferrous form, accessible to oxidation by H_2O_2.

Cyanide Increases the Rate at Which the Cell Is Killed by Fenton-driven DNA Damage—A previous study (26) reported that cyanide greatly accelerates the rate at which H_2O_2 kills *E. coli*. This result was replicated (Fig. 2*A*). Cyanide was added to the cells immediately prior to H_2O_2 exposure; after exposure, the cells were diluted and plated, and cell death was defined by the inability to form colonies. The short-term treatment with cyanide alone did not kill any cells. The phenomenon did not require protein synthesis, because the sensitization also occurred in cells treated with chloramphenicol. The dead cells formed long filaments in the hours after H_2O_2 exposure (data not shown). This behavior suggested that the damaged cells retained the metabolic capacity to grow but failed to divide because they could not replicate their DNA (27).

Maximal killing occurred when 2.5 mM H_2O_2 was used, with less killing occurring at higher concentrations (Fig. 2*B*). This pattern has been ascribed to the ability of excess H_2O_2 to scavenge the ferryl radical before it dissociates into ferric iron and the hydroxyl radical (13). As such, these kinetics suggested that the lethal cell damage arose from iron-mediated Fenton chemistry. Indeed, cells that were pretreated with the cell-permeable iron chelators desferrioxamine and *o*-phenanthroline were fully protected from the killing effects of the combined cyanide-H_2O_2 exposure (Fig. 2*A*).

The magnitude by which cyanide accelerated the killing of wild-type cells by H_2O_2 could not easily be quantified because little killing occurred in the absence of cyanide. However, using *xthA* mutants that are deficient in DNA repair, it was possible to see that cyanide caused approximately a 5-fold increase in killing rate (Fig. 2*C*). The impact of cyanide upon the killing of wild-type cells was larger than this, presumably because the number of DNA lesions produced in the absence of cyanide were few and able to be repaired, but the addition of cyanide generated enough lesions to saturate the DNA-repair process and cause death.

Cyanide Specifically Increases the Rate at Which H_2O_2 Damages Intracellular DNA—It was formally possible that cyanide accelerated cell death either by increasing the rate of DNA damage or by inhibiting the efficiency of DNA repair. However, cyanide increased the sensitivity of strains that were already genetically deficient in each of the pathways known to execute the repair of oxidized DNA. These included *recA* mutants, which lack recombinational repair capacity, as well as *xthA* and *polA* mutants, which are defective in excision and nick repair because of the absence of exonuclease III and DNA polymerase I, respectively (data not shown).

These results indicated that cyanide increased killing by accelerating the rate of DNA damage, rather than by inhibiting repair. We sought confirmation by using quantitative PCR to appraise the amount of DNA damage formed by H_2O_2. This method detects any lesions that block progression of the DNA polymerase. Data from wild-type cells confirmed that H_2O_2 created far more DNA damage when cyanide was present (Fig. 3*A*). To guard against the possibility that cyanide inhibited rapid excision repair during the period of DNA extraction, the experiment was repeated using *polA* mutants. The same re-

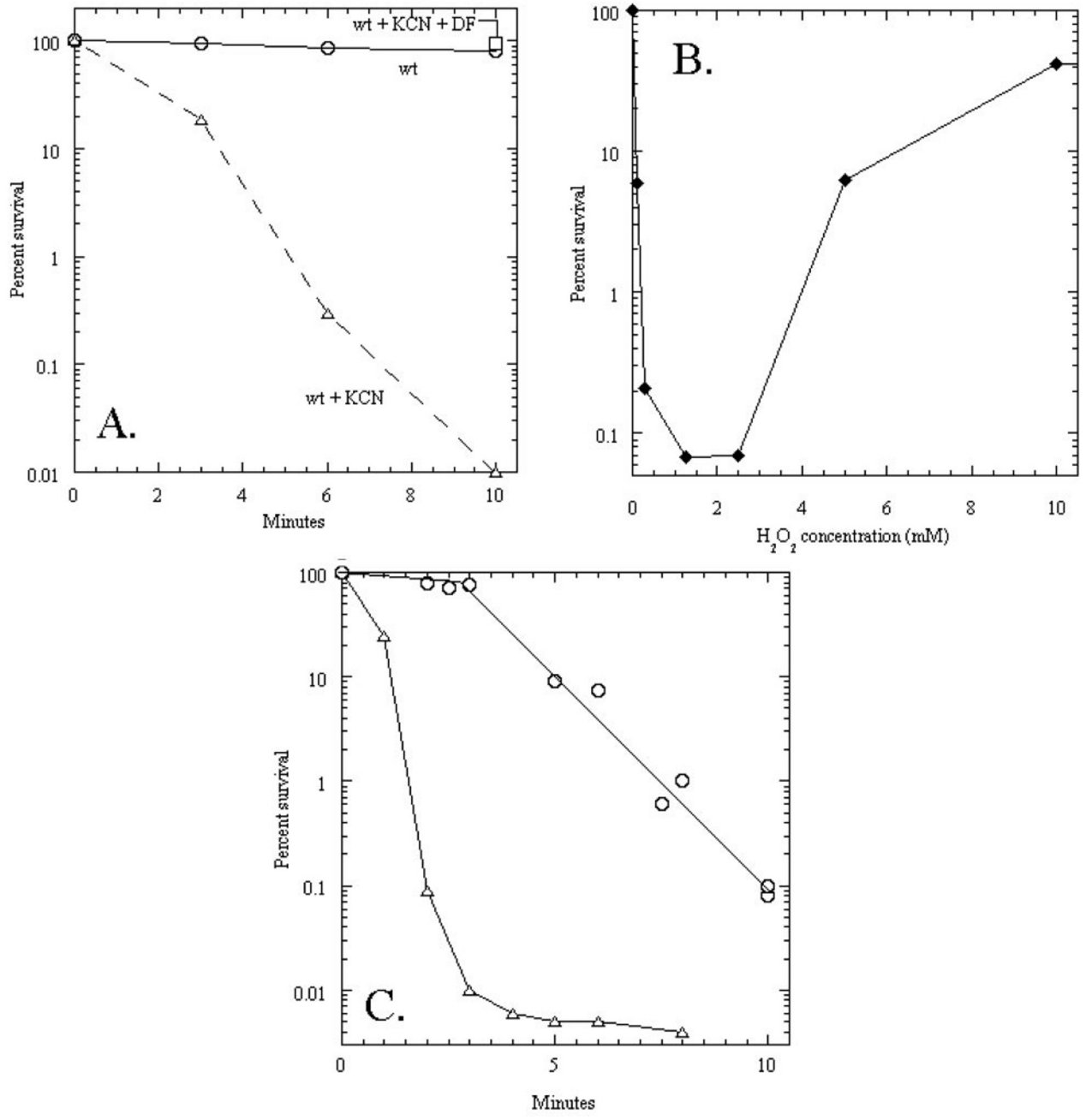

FIG. 2. **Cyanide increases the rate at which H_2O_2 kills *E. coli*.** Cells were grown in aerobic K medium and challenged with H_2O_2. Where indicated, 3 mM KCN was added to the cells 5 min prior to the addition of H_2O_2. Cyanide alone did not kill cells (not shown). *A*, wild-type (*wt*) (AN387) was challenged with 2.5 mM H_2O_2 in the absence (*circles*) or presence (*triangles*) of KCN. *Square,* 20 mM desferrioxamine (*DF*) was included 5 min before the wild-type cells were challenged in the presence of cyanide. *B*, wild-type cells were challenged with the indicated concentrations of H_2O_2 for 9 min. Less killing occurs at higher concentrations, because excess H_2O_2 scavenges the ferryl radical. *C,* AS471 (*xthA*) was challenged with 2.5 mM H_2O_2 in the absence (*circles*) or presence (*triangles*) of KCN.

sults were obtained (Fig. 3*B*). Note that cyanide itself did not damage DNA.

Cyanide Treatment Does Not Substantially Increase Free Iron Levels—According to the standard scheme of oxidative DNA damage (Reactions 2–4), only H_2O_2, iron, and a reductant are required for the generation of DNA damage. Therefore, a treatment that accelerates this process must do so by providing one of these reactants. Cyanide does not accelerate damage by affecting the intracellular concentration of H_2O_2, because the intracellular and extracellular concentrations of H_2O_2 are essentially equivalent at the doses used in this experiment (39). Although cyanide inhibits catalase, catalase is not sufficiently active to affect the intracellular H_2O_2 concentration during the period of this exposure (39). Indeed, with this challenge regime, catalase-deficient strains exhibit the same sensitivities to H_2O_2 as do wild-type cells ((34) data not shown).

EPR experiments were then performed to test whether cyanide promotes damage by increasing the amount of free iron. When cells were treated with cyanide, the free-iron pool increased only slightly, about 2-fold (from 30 to 60 μM). This amount seemed too small to explain the large effect upon oxidative damage. Furthermore, when cells were quickly washed and suspended in fresh medium, respiration resumed, and sensitivity to H_2O_2 was immediately and completely relieved. In contrast, the levels of free iron remained at 60 μM even 15 min later (data not shown). Thus the sensitivity to H_2O_2 cor-

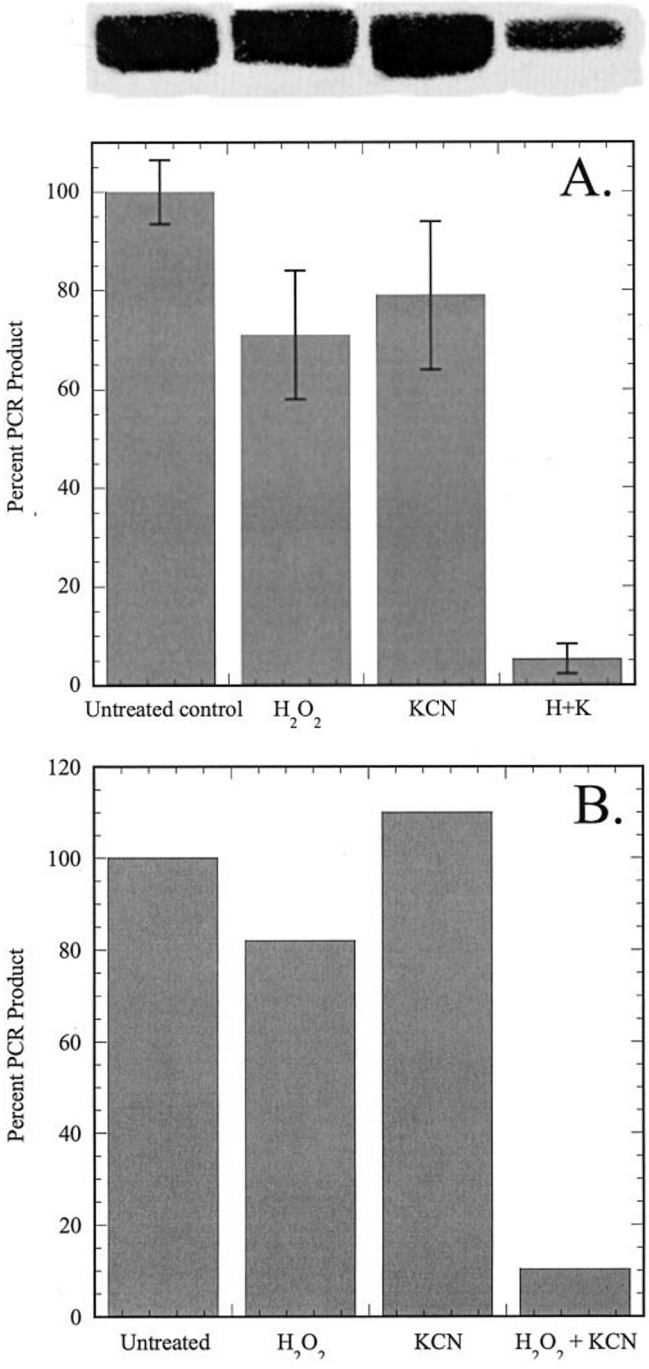

FIG. 3. **Cyanide accelerates the rate at which H_2O_2 damages DNA.** Cultures were grown aerobically in K medium and exposed to 3 mM KCN for 5 min and/or 2.5 mM H_2O_2 for 10 min, in the same manner as for the H_2O_2 killing experiments. DNA was extracted, and the ability of equimolar DNA to serve as a PCR template was measured. The amount of PCR product is proportional to the amount of undamaged template. *A*, AN387 (wild-type); mean of five experiments. Typical blots are shown *above* the corresponding lanes. *B*, ALN33 (*polA*); average results of duplicate experiments (<10% variation from mean).

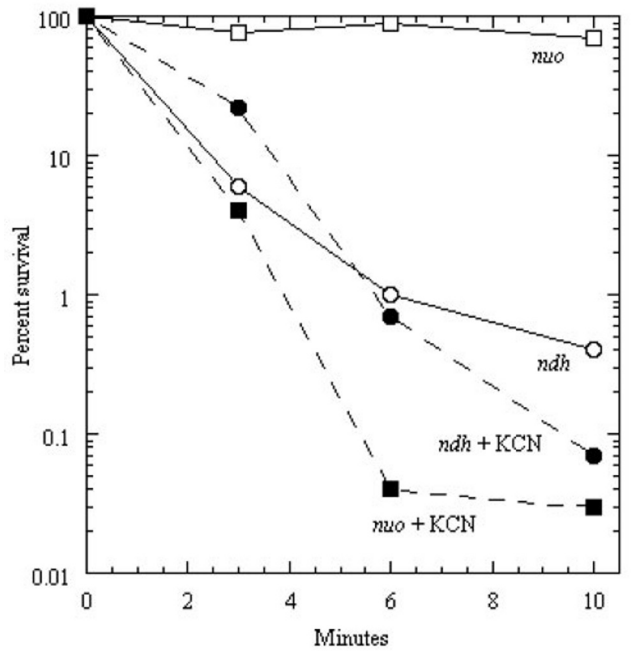

FIG. 4. **Cyanide accelerates DNA damage by blocking respiration.** JI322 (*nuo*, lacking Ndh1; *solid line*) and SLC22 (*ndh*, lacking Ndh2; *broken line*) were grown aerobically in K medium to exponential phase and challenged with 2.5 mM H_2O_2. Where indicated, 3 mM KCN was also present (*triangles*). In some experiments, there was a slight increase in killing rate when cyanide was added to the *ndh* mutant (see text).

related with respiratory inhibition and was not connected to the modest changes in free iron.

Because the major effect of cyanide is not to change the amount of H_2O_2 or free iron, we concluded that cyanide must increase the availability of the electron donor that drives the Fenton reaction (Reaction 2). Our subsequent efforts were devoted to the identification of that reductant in these cells.

Respiratory Blocks Accelerate the Fenton Reaction by Forcing the Accumulation of NADH—An earlier study (13) showed that cyanide sensitizes cells to H_2O_2 specifically because it blocks respiration. The key observation was that mutants that lacked either ubiquinone or respiratory NADH dehydrogenase activity, and therefore could not respire, were as sensitive to H_2O_2 as were cyanide-treated wild-type cells (26). The most obvious effects of these respiratory blocks are the accumulation of NADH and a dissipation of protonmotive force. Because NADH could plausibly serve as an electron donor, it was speculated that its accumulation facilitated DNA damage because NADH directly reduced free iron.

Fortunately, genetic experiments can be used to discern whether NADH concentration or pmf is the critical effector of H_2O_2 sensitivity. At the time of the earlier study, it was not realized that *E. coli* contains two respiratory NADH dehydrogenases; in fact, the Ndh^- mutant that was used in that work had mutations that eliminated both enzymes. NADH dehydrogenase I is a proton-translocating complex that is structurally and functionally analogous to the mitochondrial complex I. NADH dehydrogenase II is a non-translocating enzyme whose physiological role is to reoxidize excess NADH when the protonmotive force is high or when the NdhI activity is inadequate to handle the NADH flux. During growth on glucose both enzymes are present. In this medium the NdhII enzyme is thought to have a major role in recycling NADH, because the rate of NADH formation is very high.

We examined the sensitivity to H_2O_2 of single mutants that lacked one or the other dehydrogenase. Unlike the double mutant, both single mutants grow well in aerobic medium. With glucose as the carbon source, NADH and ATP are rapidly formed by glycolysis, and NdhII probably handles the majority of the NADH respiratory flux. The *nuo* null mutants, which lacked the proton-translocating complex, exhibited wild-type resistance when cyanide was absent and, like wild-type cells, were greatly sensitized when it was added (Fig. 4). In contrast, the *ndh* mutants were hypersensitive even when cyanide was absent. The further addition of cyanide to the *ndh* mutant

TABLE II
Impact of cyanide exposure upon intracellular pools of NADH and NADPH (μM)

AN387 was grown in aerobic K medium to exponential phase, and NADH and NADPH were extracted and quantitated as described under "Materials and Methods." Where indicated, cells were treated with 3 mM cyanide and/or 2.5 mM H_2O_2 prior to measurement. Values are the average of four trials; S.D. are shown in parentheses.

	Untreated	+ KCN	Increase
			-fold
NADH			
No H_2O_2	52 (± 12)	830 (± 250)	16
+ H_2O_2	20 (± 9.9)	330 (± 95)	16
NADPH			
No H_2O_2	770 (± 360)	960 (± 400)	1.2
+ H_2O_2	420 (± 150)	680 (± 300)	1.6

increased sensitivity slightly or not at all (Fig. 4), confirming that the effect of cyanide upon DNA damage is solely mediated by its inhibition of respiration.

Thus the impact of cyanide could be mimicked by the lack of the NADH dehydrogenase whose purpose is to reoxidize NADH but not by the lack of the pmf-generating dehydrogenase. We inferred from these results that the effect of the respiratory block on H_2O_2 sensitivity was not due to a loss of protonmotive force. Instead, it likely resulted from an accumulation of NADH. Direct measurements determined that cyanide treatment increased intracellular NADH pools 16-fold (Table II). When cells were additionally exposed to H_2O_2 (to mirror the cell-killing protocol), the NADH levels were diminished, possibly from damage to glucose catabolic enzymes; but they were again far higher with cyanide present. In contrast, the intracellular concentration of NADPH, which in growing cells is more abundant than NADH, increased less than 2-fold.

We were initially surprised to observe that cells did not become sensitive to H_2O_2 when they were challenged anaerobically, despite the presumptive lack of respiration. However, by using respiratory vesicles we determined that in the absence of oxygen H_2O_2 serves as an alternative substrate for both cytochrome oxidases *o* and *d* (see under "Materials and Methods"). Thus, during H_2O_2 challenge, anaerobic cells respire. Indeed, sensitivity was conferred either by mutations that eliminated cytochrome oxidases or by the addition of cyanide (data not shown). This result emphasizes that the sensitization by respiratory blocks does not require disruption of central metabolism, which proceeds normally in anaerobic cells. Our data indicated that the Fenton reaction is rapid whenever NADH levels are high. This result seemed to support the simple model (26) that NADH drives DNA damage by directly reducing ferric iron.

NADH Is Unlikely to be the Direct Reductant of Free Iron—The free iron that participates in Fenton reactions *in vivo* is probably bound to a variety of biomolecules, including DNA. For this reason workers have regarded small molecules, such as superoxide, NAD(P)H and glutathione, as the most probable electron donors, because these electron carriers are more likely than redox enzymes to be able to make electronic contact with iron atoms that are affixed to the surfaces of proteins, membranes, and nucleic acids. As mentioned, both experimental and quantitative arguments indicate that superoxide is not a predominant iron reductant *in vivo* (40). We therefore compared the abilities of NADH and glutathione to drive a Fenton-dependent DNA-damaging system *in vitro*. During incubation of 20 μM ferric iron with 400 μM NADH or 3 mM glutathione, levels similar to those seen in non-respiring cells, the reduction by glutathione (0.09 μM/min) proceeded much faster than that by NADH (0.01 μM/min). The experiment was repeated in the presence of H_2O_2 and bacteriophage λ, and the loss of phage infectivity was measured. Again, glutathione drove the Fenton reaction far more effectively than did NADH (data not shown). This result implied that NADH must be a comparatively insignificant direct reductant of free iron, even in cells in which the NADH pool is enlarged by respiratory blocks. Therefore, electron flow from NADH to iron must be mediated by an intermediate carrier.

Glutathione, Thioredoxin, Ferredoxin, and NADPH Are Not Responsible for Iron Reduction in Non-respiring Cells—NADH is the ultimate source of electrons for all intracellular reductants, so increases in the level of NADH could elevate the levels of these other reductants, too. They, or electron carriers downstream of them, might directly reduce iron.

When the concentration of reduced glutathione was measured in untreated and cyanide-exposed cells, virtually no difference was detected (2.4 *versus* 2.6 mM). Furthermore, like wild-type cells, *gshA* mutants were greatly sensitized to H_2O_2 by the addition of cyanide, with >80% survival when challenged without cyanide and <1% survival when challenged with it. Thus glutathione is not involved in cyanide-accelerated oxidative DNA damage. Similar experiments showed that neither thioredoxin nor ferredoxin was required for sensitivity (data not shown).

NADPH is the conduit for electron flow from NADH to biosynthetic pathways and to electron-carrying proteins, such as thioredoxin. One can exploit mutations to sever the connections between the NADPH and NADH pools, so that respiratory blocks no longer affect the NADPH level. NADPH is primarily formed in *E. coli* in two ways: by oxidation of glucose 6-phosphate via the pentose-phosphate shunt, and by the action of the pmf-driven transhydrogenase (32). Because flux through the pentose-phosphate shunt is allosterically controlled by NAD^+, and the transhydrogenase uses NADH as a substrate, we suspected that cyanide caused the moderate increase in NADPH titers by increasing the turnover of one or both of these processes. We therefore generated a mutant that lacked both transhydrogenase and glucose-6-phosphate dehydrogenase, the first enzyme in the pentose-phosphate pathway. As hoped, the NADPH levels of this strain were unusually low (~70 μM) and did not rise at all when cyanide was added, even though NADH pools rose 12-fold, from 35 to 430 μM. Yet cyanide still increased sensitivity to H_2O_2; 90% survived a 6-min H_2O_2 challenge without it but only 0.5% survived a challenge in its presence. Thus we concluded that neither NADPH nor any of the processes that derive electrons from it are responsible for reducing free iron in cyanide-treated cells.

Flavin Reductase Drives DNA Damage in Non-respiring Cells—The only other small electron carrier of which we are aware is free flavin. Whereas $FADH_2$ most commonly serves as a redox cofactor when it is tightly bound to enzymes, free $FADH_2$ is used as a co-substrate for monooxygenase reactions (42–46), including several from *E. coli* strains (28, 47, 48). Indirect evidence has suggested that $FADH_2$ may also assist in the activation of ribonucleotide reductase (33). *E. coli* contains an enzyme, called flavin reductase, that transfers electrons efficiently from NADH to free FAD (49).

We observed that mutants that lacked the structural gene for flavin reductase, *fre*, exhibited normal H_2O_2 sensitivity (Fig. 5*A*). However, in contrast to all other strains, these mutants did not become very sensitive to H_2O_2 when cyanide was added. When the *fre* mutation was transduced into unrelated *E. coli* K-12 strains, they too became resistant to the synergistic effects of cyanide and H_2O_2. Complementation with *fre* on a plasmid restored wild-type behavior (Fig. 5*A*). The *fre* mutation also protected *ndh* mutants from H_2O_2 (Fig. 5*B*). Interestingly, strains that overproduced flavin reductase anaerobically were

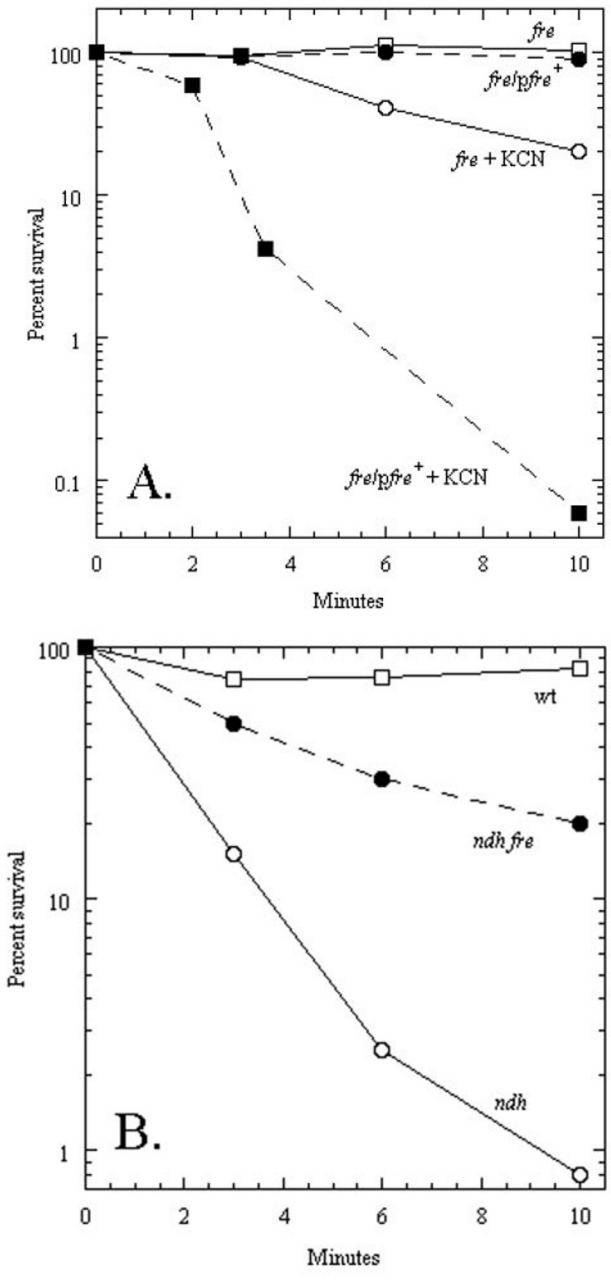

FIG. 5. **A defect in flavin reductase diminishes the rate of oxidative damage in non-respiring cells.** *A*, ALN2 (*fre*) and ALN4 (*fre*/pFN3[*fre*$^+$]) were grown to exponential phase in aerobic K medium and challenged with 2.5 mM H_2O_2. IPTG (1 mM) was added to cells two generations before the challenge in order to induce abundant synthesis of flavin reductase from the plasmid Ptac promoter. *B*, SLC22 (*ndh*) and ALN18 (*ndh fre*) were grown to exponential phase and challenged with H_2O_2. *wt*, wild type.

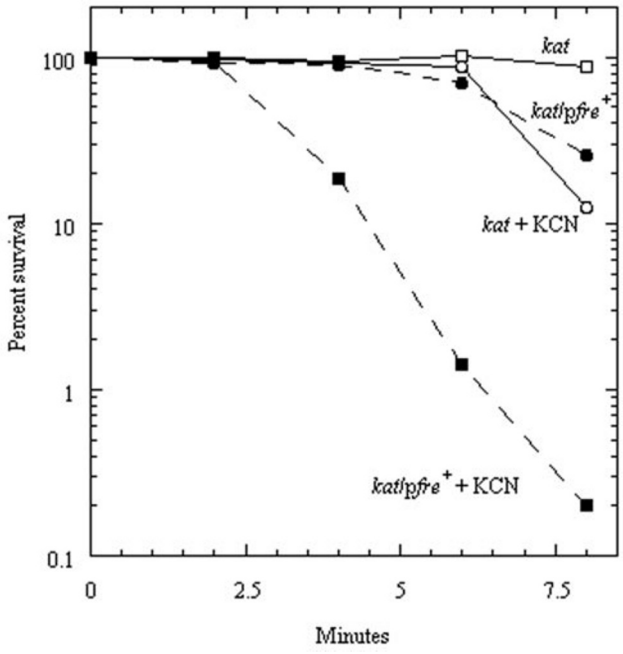

FIG. 6. **Overexpressed flavin reductase accelerates damage in the absence of oxygen.** JI367 (Kat$^-$) and ALN30 (Kat$^-$/pFN3[*fre*$^+$]) were grown and challenged in oxygen-free K medium with 3 mM KCN and 2.5 mM H_2O_2 as indicated. Synthesis of flavin reductase was induced by the addition of 1 mM IPTG two doublings prior to the H_2O_2 challenge.

even more sensitive to H_2O_2 than were wild-type cells (Fig. 6). (In this experiment catalase-deficient mutants were used in order to prevent O_2 formation from the decomposition of H_2O_2.)

In all experiments the *fre* mutation abolished most, but not all, of the sensitization that cyanide causes. The small *fre*-independent sensitization may be caused by the 2-fold increase in free-iron concentration, which persisted in *fre* mutants (data not shown).

$FADH_2$ That Is Produced by Flavin Reductase Efficiently Transfers Electrons to Ferric Iron—The genetic and metabolic data suggested that respiratory blocks enlarge the NADH pools and thereby accelerate the rate at which flavin reductase transfers electrons to free flavins, which then reduce free iron. This scheme could only be true if normal NADH pools were insufficient to saturate flavin reductase activity. In fact, the apparent K_m value of the enzyme for NADH is unusually high, 300 μM (50). This substantially exceeds the NADH concentration of respiring cells. Michaelis-Menten calculations suggest that an increase from 20 to 330 μM NADH (Table II) could potentially increase the flux through flavin reductase by 8.4-fold. Thus the kinetic properties of the enzyme conform with the impact of the physiological inhibitor.

Flavin reductase was purified to homogeneity. In the presence of NADH and FAD, Fre rapidly catalyzed the reduction of ferric iron (Fig. 7*A*). The reaction rate was far lower when iron was incubated with glutathione or with NADH in the absence of the enzyme. When H_2O_2 and plasmid DNA were added to the flavin reduction system, the DNA was efficiently damaged (Fig. 7*B*).

The presumptive reaction series in this system is shown in Reactions 5–8 as follows:

$$NADH + FAD^+ \rightarrow NAD^+ + FADH_2$$

REACTION 5

$$FADH_2 + Fe^{3+} \rightarrow FADH^\cdot + Fe^{2+} + H^+$$

REACTION 6

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^\cdot + OH^-$$

REACTION 7

$$HO^\cdot + DNA \rightarrow H_2O + \text{oxidized DNA}$$

REACTION 8

Flavin reductase catalyzes the Reaction 5; Reactions 6–8 are spontaneous. The flavosemiquinone that is generated in Reaction 7 could in principle reduce another iron atom; however, in an aerobic environment it would more likely react with oxygen to

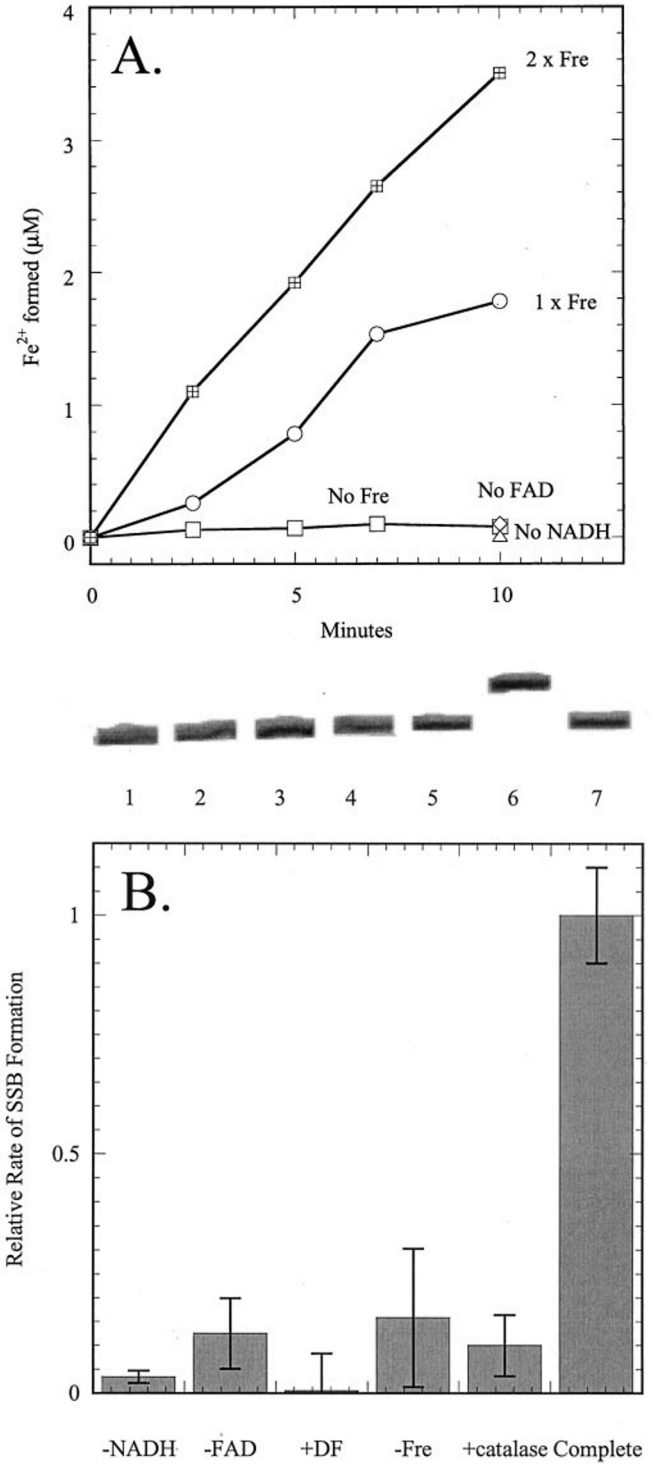

FIG. 7. **Flavin reductase efficiently reduces free iron *in vitro*.** *A,* production of ferrous iron. Complete reactions contained 10 μM ferric chloride, 300 μM NADH, 15 μM FAD, and 20 milliunits of purified flavin reductase in a final volume of 1.5 ml of 20 mM Tris, pH 7.4. All reagents were anaerobic. *B*, generation of strand breaks in plasmid DNA *in vitro*. The anaerobic reactions contained 200 μM NADH, 15 μM FAD, 10 μM $FeCl_3$, 10 milliunits of flavin reductase, 10 ng of DNA, and 200 μM H_2O_2. Shown above is a typical gel corresponding directly to the reactions graphed below. *Lane 1*, NADH omitted; *lane 2*, FAD omitted; *lane 3*, desferrioxamine added; *lane 4*, Fre omitted; *lane 5*, catalase added; *lane 6*, complete reaction; *lane 7*, control DNA only. Where indicated, 3 mM glutathione was substituted for NADH, FAD, and flavin reductase.

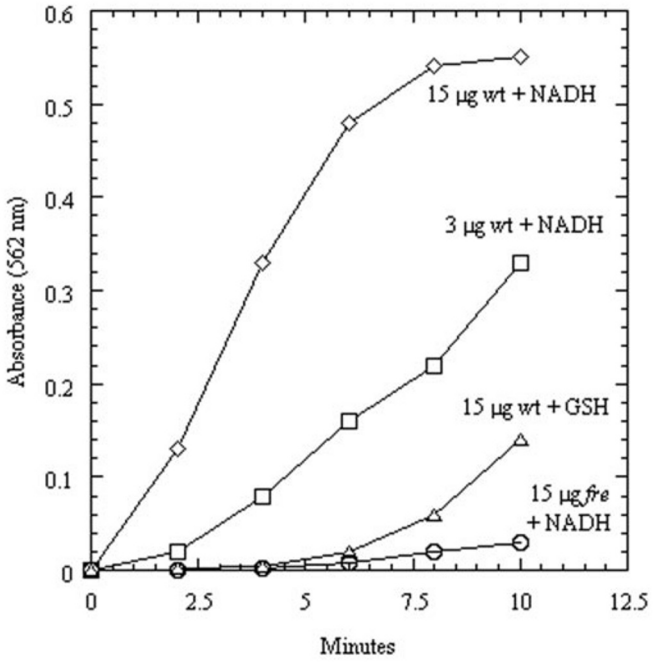

FIG. 8. **Production of ferrous iron by flavin reductase in crude extracts.** Cell extracts of AN387 (wild-type, *wt*) and ALN2 (*fre*) were prepared from exponential phase cultures in aerobic K medium. $FeCl_3$ (10 μM), FAD (15 μM), and either NADH (400 μM, *solid lines*) or GSH (400 μM, *broken line*), as indicated, were added to 3 or 15 μg of total protein in 1.5 ml of anaerobic water, and the progress of iron reduction was monitored as described under "Materials and Methods." No significant reaction occurred in the absence of reductant or when FAD was omitted from the NADH-driven reactions (not shown).

generate superoxide, a proton, and a fully oxidized flavin.

The titer of Fre in wild-type cells is sufficiently high that cell extracts, NADH, and FAD efficiently reduce ferric iron (Fig.8). The same reaction was much slower in extracts from a *fre* mutant. Fre provides about 80% of the flavin reductase activity of wild-type extracts; the low residual activity is presumably due to the redox enzymes that reduce free flavins (51).

Because flavins autoxidize in aerobic solution, these reactions were all conducted anaerobically to confirm that superoxide need not mediate the electron transfer from flavin to iron. Superoxide does not mediate the effect of flavin reductase *in vivo* either, because it accelerated damage even in anaerobic cells (Fig. 6).

Thus flavin reductase constitutes the most efficient *in vitro* iron reduction system that we have yet found. Our results parallel those of Coves and Fontecave (52), who recovered flavin reductase when they purified an activity that efficiently reduced iron-siderophore chelates.

Flavin Reductase Promotes DNA Damage in Vivo Only When Respiratory Blocks Increase the Pool of NADH—We wanted to determine whether flavin reductase was also responsible for the DNA damage that occurs when H_2O_2 is added to cells that are respiring normally. The rate of killing is not easily quantified in wild-type cells, which efficiently repair the lesions. The *fre* mutation was therefore transduced into repair-defective *xth* and *recA* strains, in which killing by H_2O_2 is substantial. The mutation did not diminish the rate of cell death when H_2O_2 was added in the absence of cyanide (Fig. 9). The mutation did, however, diminish the acceleration of killing that occurred when cyanide was added. Therefore, flavin reductase is the preponderant source of electrons for free iron only when the pool of NADH is large enough to satisfy its high K_m. In respiring cells flavin reductase is less active, and another, slower pathway of iron reduction predominates. That other pathway has not been identified.

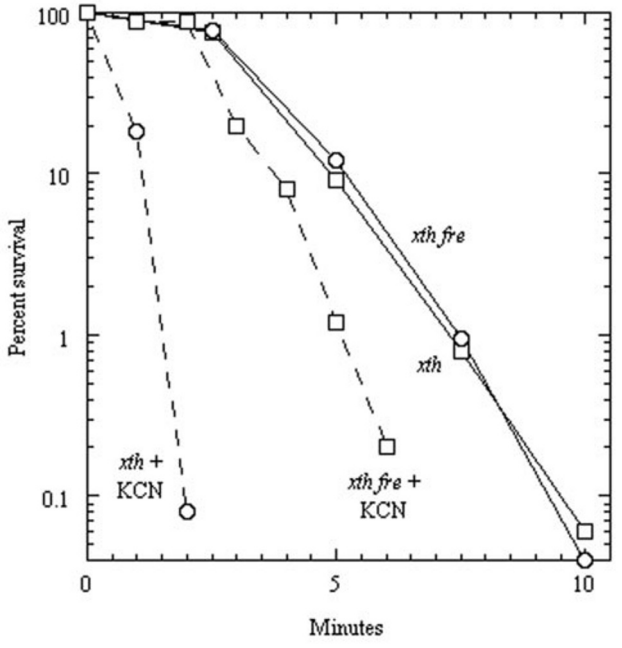

FIG. 9. **Flavin reductase only accelerates oxidative DNA damage in non-respiring cells.** Mutants lacking exonuclease III were grown aerobically and challenged with 2.5 mM H_2O_2 in the absence or presence of 3 mM KCN.

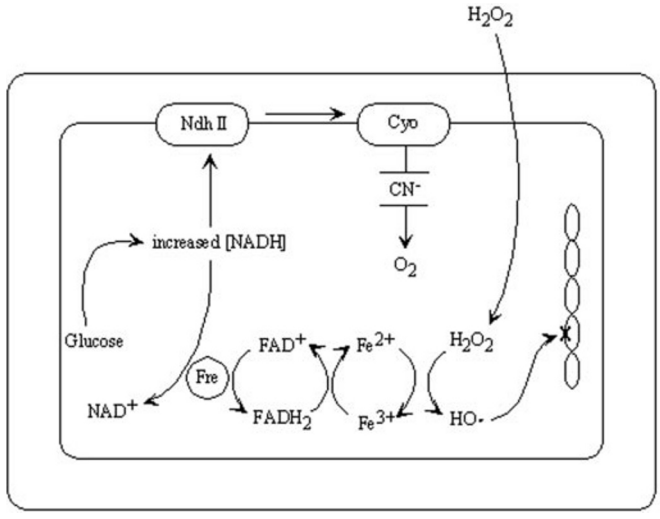

FIG. 10. **Pathway of electrons from NADH to H_2O_2 in non-respiring cells.** NADH only accumulates in sufficient quantity for this pathway of iron reduction to predominate when respiration is blocked (*top*). Electron transfer from $FADH_2$ to ferric iron is univalent; in aerobic cells, the resultant flavosemiquinone probably transfers the second electron to oxygen (65).

DISCUSSION

Free $FADH_2$ Is an Efficient Reductant of Free Iron—These results indicate that the rate of oxidative DNA damage depends upon how quickly free iron is reduced, and they show that free $FADH_2$ can be an efficient reductant. In non-respiring *E. coli*, Fenton chemistry occurs when electrons are transferred from the enlarged NADH pools to FAD, from $FADH_2$ to iron, and finally from iron to H_2O_2 (Fig. 10). This adventitious pathway resembles enzymic electron-transport chains, which also employ iron to break the oxygen-oxygen bond and flavins to bridge electron flow from the two-electron nicotinamide donor to the metal center acceptor.

Metal catalysts may be required for electron transfer to H_2O_2 because they weaken the oxygen-oxygen bond. Destabilization is presumably accomplished by interaction of an H_2O_2 oxygen atom with the metal *d*-orbitals; this is suggested by the fact that, *in vivo* and *in vitro*, the immediate product of the Fenton reaction is a ferryl radical, FeO^{2+}, rather than a free hydroxyl radical (13, 53). An "organic Fenton reaction" reported recently (54) appeared to be exceptional, but further study has suggested that the reaction may proceed by addition of H_2O_2 to an unusual quinone reductant, rather than by direct electron transfer.

$FADH_2$ efficiently reduces iron because reduced flavins are facile univalent reductants. This trait is a consequence of the low reduction potential of the flavosemiquinone/dihydroflavin pair (−0.15 V) (55). Enzymes that transfer electrons from divalent donors to internal metal centers, such as succinate dehydrogenase, typically use flavins to do so; therefore, it is not surprising that $FADH_2$ also efficiently reduces free iron. In contrast, the $NADH^+$/NADH potential is +0.93 V (56) and that of the glutathione $GS^{\cdot}$/GSH couple is +0.90 V (57). Although it is formally true that subsequent reactions of these radical products can pull the initial reactions forward, these high reduction potentials apparently establish a kinetic barrier that prohibits either NADH or GSH from being as effective a univalent reductant as is $FADH_2$.

Adventitious Redox Chemistry May be Surprisingly Common—Our current understanding holds that a series of five adventitious univalent electron transfers comprises the process of DNA damage; molecular oxygen oxidizes reduced flavoproteins to generate superoxide and H_2O_2, the superoxide then oxidizes iron-sulfur clusters and releases free iron, free $FADH_2$ reduces the free iron, the reduced iron transfers an electron to H_2O_2, and the resultant hydroxyl radical abstracts an electron from DNA. It may seem surprising that a nonenzymatic process with so many steps should progress with enough efficiency to have an impact upon the cell, particularly because the intermediates can be drawn off by side reactions and scavenging processes. However, the rate constants of the individual reactions are very high, with the lowest ($k = 76\ M^{-1}\ s^{-1}$) being that of the Fenton reaction. Univalent redox reactions often have low rearrangement energies and therefore proceed more readily than other adventitious reactions, requiring primarily that the donor and acceptor be brought into electronic contact and that the reduction potentials favor the reaction. Investigators have long taken advantage of this principle by using small dyes as efficient artificial substrates for redox enzymes. Oxidative stress is an unfortunate manifestation of the same idea; oxygen species are good oxidants and are small enough that they cannot easily be excluded from the active sites of enzymes. Their ability to intercept electrons is a problem that nature cannot easily solve.

What Is the Role of Flavin Reductase?—The ability of Fre to reduce free flavins serves no obvious physiological purpose. Mutants that lack *fre* are sensitive to hydroxyurea, which deactivates ribonucleotide reductase; because reduced free flavins can reactivate the enzyme *in vitro*, it has been suggested that the production of diffusible $FADH_2$ might be the real function of Fre (33). However, this enzyme efficiently reduces the free FAD pool only if NADH accumulates to unusually high levels, and those levels are achieved only when respiration is blocked. It is hard to understand why an enzyme would have such a high K_m that it could not turn over rapidly under normal physiological conditions.

An explanation may be suggested by the fact that the *fre* gene exhibits homology to the flavin reductases that provide $FADH_2$ to monooxygenases. In at least one case, kinetic data indicate that the partner reductase and monooxygenase physically interact (58). If so, $FADH_2$ may be directly channeled between the enzymes, and the production of free $FADH_2$ by

small amounts of uncomplexed reductase may be adventitious. That scenario might explain the unfavorable kinetics of NADH binding by isolated Fre.

Furthermore, it is notable that the overproduction of Fre increased the rate of oxidative DNA damage above that of wild-type cells. That observation suggests that the normal metabolic contribution of Fre is not sufficient to fully sensitize cells, again as if it is the aberrant over-reduction of the free FAD pool that causes sensitivity.

The *fre* gene lies immediately downstream of *ubiD*, which encodes an enzyme in the ubiquinone biosynthetic pathway. Aerobic ubiquinone biosynthesis involves oxygenation steps whose enzymes have not been genetically defined (59), and we wondered whether *fre* might encode a reductase that provides $FADH_2$ to one of them. However, other workers (60) recently demonstrated that ubiquinone synthesis is normal in *fre* strains. To guard against the possibility that a suppressor mutation had arisen in those *fre* mutants, we transduced the *fre* mutation anaerobically into fresh backgrounds and then re-checked the aerobic respiration rate. Our data support the conclusion that *fre* does not affect ubiquinone synthesis. For now its role remains uncertain.

What Is the Predominant Reductant of Free Iron Under Usual Biological Conditions?—Reaction 3 dictates that the rate at which H_2O_2 damages DNA depends directly upon the concentration of free ferrous iron. This idea underpinned much early work on superoxide, which was thought to damage cells by serving as a reductant of free iron and thereby driving the Fenton reaction. It turned out that superoxide is too scarce to do so (23, 40). However, the results of this study confirm that when H_2O_2 concentrations are high, the rate of iron reduction does determine the pace of oxidative DNA damage. Furthermore, we have found that free $FADH_2$ can be an important intracellular iron reductant, although its concentration may be high in *E. coli* only when respiration has been blocked.

We have recently determined that the synergistic killing of *E. coli* by NO and H_2O_2 (62) is primarily due to the inhibition of cytochrome oxidase by NO and the consequent flux through flavin reductase, leading to rapid DNA damage.[2] Further experiments will be necessary to reveal whether this damage pathway is a significant killing mechanism inside phagocytes.

We remain interested in identifying the electron donor that drives DNA damage under normal conditions, when cells respire freely. Whereas our data indicate that flavin reductase itself is not involved (Fig. 9), it remains possible that $FADH_2$ that is generated by other enzymes is the predominant reductant. Many redox enzymes can adventitiously reduce free flavins, albeit at a lower rate than does Fre. Sulfite reductase, for example, has a significant flavin reductase activity (2, 63). Alternatively, in the absence of much free $FADH_2$, other small electron carriers like thiols may become the predominant donors. Further work will be necessary to resolve this issue.

Whatever the reductant(s), the cytosolic compartment of *E. coli* is a sufficiently reducing environment that free iron accumulates in the ferrous form, despite the oxidizing force of molecular oxygen and endogenous H_2O_2. For this reason, free iron is poised to participate in Fenton reactions and thereby contribute to mutation rates. Interestingly, this is not true of *Saccharomyces cerevisiae*, whose pools of free iron are predominantly oxidized (64). It is possible that in yeast this iron is localized in a compartment that lacks univalent reductants, thereby minimizing the impact of the iron upon oxidative DNA damage.

[2] A. N. Woodmansee and J. A. Imlay, manuscript in preparation.

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REFERENCES

1. Massey, V., Strickland, S., Mayhew, S. G., Howell, L. G., Engel, P. C., Matthews, R. G., Schuman, M., and Sullivan, P. A. (1969) *Biochem. Biophys. Res. Commun.* **36,** 891–897
2. Messner, K. R., and Imlay, J. A. (1999) *J. Biol. Chem.* **274,** 10119–10128
3. Massey, V. (1994) *J. Biol. Chem.* **36,** 22459–22462
4. Flint, D. H., Tuminello, J. F., and Emptage, M. H. (1993) *J. Biol. Chem.* **268,** 22369–22376
5. Storz, G., and Imlay, J. A. (1999) *Curr. Opin. Microbiol.* **2,** 188–194
6. Carlioz, A., and Touati, D. (1986) *EMBO J.* **5,** 623–630
7. Seaver, L. C., and Imlay, J. A. (2001) *J. Bacteriol.* **183,** 7173–7181
8. Brawn, K., and Fridovich, I. (1981) *Arch. Biochem. Biophys.* **206,** 414–419
9. Lesko, S. A., Lorentzen, R. J., and Ts'o, P. O. P. (1980) *Biochemistry* **19,** 3023–3028
10. Hutchinson, F. (1985) *Prog. Nucleic Acids Res.* **32,** 116–154
11. Aruoma, O. I., Halliwell, B., Gajewski, E., and Dizdaroglu, M. (1989) *J. Biol. Chem.* **264,** 20509–20512
12. Meneghini, R. (1997) *Free Radic. Biol. Med.* **23,** 783–792
13. Imlay, J. A., and Linn, S. (1988) *Science* **240,** 640–642
14. Nascimento, A. L. T. O., and Meneghini, R. (1995) *Carcinogenesis* **16,** 1335–1338
15. Touati, D., Jacques, M., Tardat, B., Bouchard, L., and Despied, S. (1995) *J. Bacteriol.* **177,** 2305–2314
16. Keyer, K., and Imlay, J. A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 13635–13640
17. Walling, C. (1975) *Acc. Chem. Res.* **8,** 125–131
18. McCord, J. M., and Day, E. D., Jr. (1978) *FEBS Lett.* **86,** 139–142
19. Imlay, J. A., and Fridovich, I. (1991) *J. Biol. Chem.* **266,** 6957–6965
20. Tyler, D. D. (1975) *Biochem. J.* **147,** 493–504
21. Bielski, B. H. J., Cabelli, D. E., and Arudi, R. L. (1985) *J. Phys. Chem. Ref. Data* **14,** 1041–1062
22. Farr, S. B., D'Ari, R., and Touati, D. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 8268–8272
23. Liochev, S. I., and Fridovich, I. (1994) *Free Radic. Biol. Med.* **16,** 29–33
24. Rowley, D. A., and Halliwell, B. (1982) *FEBS Lett.* **142,** 39
25. Winterbourn, C. C. (1979) *Biochem. J.* **182,** 625–628
26. Imlay, J. A., and Linn, S. (1988) *Science* **240,** 1302–1309
27. Imlay, J. A., and Linn, S. (1986) *J. Bacteriol.* **166,** 519–527
28. Xun, L., and Sandvik, E. R. (2000) *Appl. Environ. Microbiol.* **66,** 481–486
29. Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
30. Demple, B., Halbrook, J., and Linn, S. (1983) *J. Bacteriol.* **153,** 1079–1082
31. Wolf, R. E., Jr., Prather, D. M., and Shea, F. M. (1979) *J. Bacteriol.* **139,** 1093–1096
32. Hanson, R. L., and Rose, C. (1980) *J. Bacteriol.* **141,** 401–404
33. Fontecave, M., Eliasson, R., and Reichard, P. (1987) *J. Biol. Chem.* **262,** 12325–12331
34. Imlay, J. A., and Linn, S. (1987) *J. Bacteriol.* **169,** 2967–2976
35. Yakes, F. M., and Houten, B. V. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 514–519
36. Lawley, P. D., and Thatcher, C. J. (1970) *Biochem. J.* **116,** 693–707
37. Greenberg, J. T., and Demple, B. (1986) *J. Bacteriol.* **168,** 1026–1029
38. Yegorov, D. Y., Kozlov, A. V., Azizova, O. A., and Vladimirov, Y. A. (1993) *Free Radic. Biol. Med.* **15,** 565–574
39. Seaver, L. C., and Imlay, J. A. (2001) *J. Bacteriol.* **183,** 7182–7189
40. Keyer, K., Gort, A. S., and Imlay, J. A. (1995) *J. Bacteriol.* **177,** 6782–6790
41. Linn, S., and Imlay, J. A. (1987) *J. Cell Sci.* (suppl.) **6,** 289–301
42. Lei, B., Liu, M., Huang, S., and Tu, S. C. (1994) *J. Bacteriol.* **176,** 3552–3558
43. Thibaut, D., Ratet, N., Bisch, D., Faucher, D., Debussche, L., and Blanche, F. (1995) *J. Bacteriol.* **177,** 5199–5205
44. Xu, Y., Mortimer, M. W., Fisher, T. S., Kahn, M. L., Brockman, F. J., and Xun, L. (1997) *J. Bacteriol.* **179,** 1112–1116
45. Witschel, M., Nagel, S., and Egli, T. (1997) *J. Bacteriol.* **179,** 6937–6943
46. Lei, B., and Tu, S. C. (1996) *J. Bacteriol.* **178,** 5699–5705
47. Eichhorn, E., van der Ploeg, J. R., and Leisinger, T. (1999) *J. Biol. Chem.* **274,** 26639–26646
48. Galan, B., Diaz, E., Prieto, M. A., and Garcia, J. L. (2000) *J. Bacteriol.* **182,** 627–636
49. Spyrou, G., Haggard-Ljungquist, E., Krook, M., Jornval, H., Nilsson, E., and Reichard, P. (1991) *J. Bacteriol.* **173,** 3673–3679
50. Niviere, V., Fieschi, F., Decout, J., and Fontecave, M. (1999) *J. Biol. Chem.* **274,** 18252–18260
51. Fontecave, M., Coves, J., and Pierre, J. L. (1994) *Biometals* **7,** 3–8
52. Coves, J., and Fontecave, M. (1993) *Eur. J. Biochem.* **211,** 635–641
53. Rush, J. D., Maskos, Z., and Koppenol, W. H. (1990) *Methods Enzymol.* **186,** 148
54. Zhu, B.-Z., Zhao, H.-T., Kalyanaraman, B., and Frei, B. (2002) *Free Radic. Biol. Med.* **32,** 465–473
55. Meisel, D., and Neta, P. (1975) *J. Phys. Chem.* **79,** 2459–2461
56. Carlson, B. W., Miller, L. L., Neta, P., and Grodkowski, J. (1984) *J. Am. Chem. Soc.* **106,** 7233–7239
57. Koppenol, W. H. (1993) *Free Radic. Biol. Med.* **14,** 91–94
58. Jeffers, C. E., and Tu, S. (2001) *Biochemistry* **40,** 1749–1754
59. Meganathan, R. (1996) in *Escherichia coli and Salmonella* (Neidhardt, F. C., Curtiss, R., III, Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., and Umbarger, H. E., eds) pp.

642–656, American Society for Microbiology, Washington, D. C.
60. Poon, W. W., Davis, D. E., Ha, H. T., Jonassen, T., Rather, P. N., and Clarke, C. F. (2000) *J. Bacteriol.* **182,** 5139–5146
61. Fieschi, F., Niviere, V., Frier, C., Decout, J. L., and Fontecave, M. (1995) *J. Biol. Chem.* **270,** 30392–30400
62. Pacelli, R., Wink, D. A., Cook, J. A., Krishna, M. C., DeGraff, W., Friedman, N., Tsokos, M., Samuni, A., and Mitchell, J. B. (1995) *J. Exp. Med.* **182,** 1469–1479
63. Coves, J., Niviere, V., Eschenbrenner, M., and Fontecave, M. (1993) *J. Biol. Chem.* **268,** 18604–18609
64. Srinivasan, C., Liba, A., Imlay, J. A., Valentine, J. S., and Gralla, E. B. (2000) *J. Biol. Chem.* **275,** 29187–29192
65. Muller, F. (1987) *Free Radic. Biol. Med.* **3,** 215–230
66. Au, D. C.-T., Green, G. N., and Gennis, R. B. (1984) *J. Bacteriol.* **157,** 122–125