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In vivo kinetics of a redox-regulated transcriptional switch

(SoxR protein/oxidative stress/iron-sulfur clusters/gene expression/mRNA stability)

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ABSTRACT SoxR is a transcription activator governing a cellular response to superoxide and nitric oxide in *Escherichia coli*. SoxR protein is a homodimer, and each monomer has a redox-active [2Fe–2S] cluster. Oxidation and reduction of the [2Fe–2S] clusters can reversibly activate and inactivate SoxR transcriptional activity. Here, we use electron paramagnetic resonance spectroscopy to follow the redox-switching process of SoxR protein *in vivo*. SoxR [2Fe–2S] clusters were in the fully reduced state during normal aerobic growth, but were completely oxidized after only 2-min aerobic exposure of the cells to superoxide-generating agents such as paraquat. The oxidized SoxR [2Fe–2S] clusters were rapidly re-reduced *in vivo* once the oxidative stress was removed. The *in vivo* kinetics of SoxR [2Fe–2S] cluster oxidation and reduction exactly paralleled the increase and decrease of transcription of *soxS*, the target gene for SoxR. The kinetic analysis also revealed that an oxidative stress-linked decrease in *soxS* mRNA stability contributes to the rapid attainment of a new steady state after SoxR activation. Such a redox stress-related change in *soxS* mRNA stability may represent a new level of biological control.

Reactive oxygen species are produced as the by-products of aerobic metabolism (1) and mediate the toxicity of many environmental agents (2). Primary reactive products such as superoxide anion (O_2^-) and H_2O_2 can damage all cellular components and thus exert oxidative stress (3). Cells respond to the challenges of oxidative stress by activating genetic responses. These responses include important defense activities that have been quite well defined in bacteria (4). The *oxyR* regulon, activated by H_2O_2 (5) or nitrosothiols (6), increases the levels of catalase, alkyl hydroperoxidase, glutathione reductase, and a protective DNA binding protein (4). A separate response is activated by superoxide-generating agents or nitric oxide: the *soxRS* regulon (7). The *soxRS* system increases expression of superoxide dismutase, glucose-6-phosphate dehydrogenase, fumarase C, aconitase, and the DNA repair enzyme endonuclease IV (4). In addition to such defenses against oxidative damage, the *soxRS* regulon activates resistance to multiple antibiotics (8) and to organic solvents and heavy metals (9). Activation of the *soxRS* regulon is also important for resistance of *Escherichia coli* to nitric oxide-generating macrophages (10).

Gene induction in the *soxRS* system occurs in two stages of transcriptional activation (11, 12). SoxS, an AraC-related protein, is the direct activator of the regulon genes by binding their promoter regions (13–15). The cellular level of SoxS is in turn controlled through the activity of SoxR (11, 12). SoxR is a homodimer that binds between the –10 and –35 elements of the *soxS* promoter, and in the activated state overcomes the suboptimal spacing of the promoter elements (16) to trigger

transcription up to 100-fold. Dimeric SoxR contains a pair of redox-active [2Fe–2S] clusters, and these allow transcriptional activity *in vitro* only when they are in the oxidized state (17, 18).

SoxR protein with reduced [2Fe–2S] centers is the predominant form present in cells prior to oxidative stress, as determined by electron paramagnetic resonance (EPR) spectroscopy of intact cells overproducing the wild-type protein (19, 20). *In vivo* EPR analysis indicates that constitutively active mutant forms of SoxR are prone to oxidation of the [2Fe–2S] centers during normal aerobic growth in the absence of redox-cycling agents. For one such mutant protein, its oxidation during normal growth is correlated with a significant shift in the redox midpoint potential that could result in an accelerated rate of oxidation (19). The EPR signal of reduced SoxR [2Fe–2S] clusters was decreased in cells treated with superoxide-generating agents, consistent with oxidation of the [2Fe–2S] centers; removal of the oxidative stress allowed the protein to be re-reduced (20). However, the rates of intracellular oxidation and reduction of SoxR protein are unknown because of the time needed to concentrate the cell samples for the published EPR measurements (19, 20).

We have now developed methods for EPR analysis of samples taken directly from cell cultures, which has allowed us to follow the kinetics of SoxR [2Fe–2S] oxidation and reduction *in vivo*. These experiments show that the electron-transfer processes involving the [2Fe–2S] centers are fast, and that *soxS* transcription is directly proportional to the amount of oxidized SoxR. The kinetic analysis also indicates that the stability of the *soxS* message is decreased during oxidative stress but quickly returns to the higher (nonstress) level when the stress is removed.

MATERIALS AND METHODS

EPR Sample Preparation and Measurement. Overnight cultures of *E. coli* strain XA90 containing the SoxR expression plasmid pK0XR or the control plasmid pKEN2 (21) were diluted 1:100 into 50 ml of Luria–Bertani medium containing 100 μ g/ml ampicillin in a 300-ml flask. The cultures were incubated at 37°C with shaking at 275 rpm for 2 h, then isopropyl β -D-thiogalactopyranoside (0.5 mM) was added and incubation continued for 1 h to induce the expression of SoxR protein before additional treatment. Where indicated, the cultures (at OD_{600} 1.0–1.2) were treated with redox-cycling agents with continued aeration by shaking at 275 rpm, or the shaking was stopped to stop aeration. Within 2 min after stopping aeration, the dissolved O_2 concentration reached <1% of that in the shaking cultures as measured with an oxygen micro-electrode (Microelectrodes, Bedford, NH). For direct EPR measurement, 0.4-ml aliquots were taken directly from the cultures, transferred to EPR tubes, and instantly frozen in a mixture containing isobutane/cyclohexane (5:1) chilled with liquid nitrogen. For concentrated cell samples,

Abbreviations: EPR, electron paramagnetic resonance; PQ, paraquat. *To whom reprint requests should be addressed. e-mail: demple@mbcrr.harvard.edu.

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cells were harvested from 50 ml of culture and resuspended in 1 ml of buffer containing 500 mM NaCl and 50 mM Hepes (pH 7.6). Aliquots (0.4 ml) of the concentrated cells were transferred to EPR tubes, and parallel 0.4-ml aliquots were analyzed for total SoxR protein using SDS/PAGE and staining followed by quantitative densitometry. Samples of purified SoxR, previously quantified by amino acid analysis, were used as the reference standard.

The X-band EPR spectra of SoxR [2Fe-2S] clusters were obtained using a Bruker (Billerica, MA) model ESP-300 equipped with an Oxford Instruments (Eynsham, U.K.) model 910 continuous flow cryostat (courtesy of J. Stubbe's laboratory, Massachusetts Institute of Technology, or P. Leslie Dutton's laboratory, University of Pennsylvania). Routine EPR measurement conditions were as follows: microwave frequency, 9.47 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 1.2 mT; sweep field, 310–370 mT; sample temperature, 30 K; receiver gain, 10^5 .

Analysis of *soxS* mRNA. One-milliliter samples of *E. coli* cultures (containing $\approx 10^9$ cells) were transferred to 1.5-ml Eppendorf tubes and immediately frozen as above. Frozen samples were quickly thawed in a 65°C water bath, and total RNA was isolated using an RNeasy Mini Kit following the manufacturer's instructions (Qiagen, Chatsworth, CA). Northern blot analysis was carried out as described (22). A *soxS*-specific probe was prepared by labeling the *EcoRI*–*HindIII* fragment of plasmid pSXS (13) using a random-primer system (Life Technologies, Gaithersburg, MD). The amount of *soxS* mRNA in Northern blot analyses was quantified using scanning densitometry (Bio Image; Millipore).

RESULTS AND DISCUSSION

In wild-type *E. coli* cells, there are relatively few copies of SoxR protein (≤ 100 molecules per cell; E. Hidalgo and B.D.,

unpublished data), so that direct spectroscopic observation of SoxR *in vivo* requires overproduction of the protein. Expression of SoxR to $\approx 5\%$ of cellular protein did not significantly affect cell growth, but did provide a window to monitor the redox state of SoxR [2Fe-2S] clusters *in vivo* (19, 20). After concentration, such cells yielded a clear EPR spectrum characteristic of reduced SoxR [2Fe-2S] clusters (Fig. 1A) that was identical to that for purified, reduced SoxR (21, 23, 24). No EPR signal near $g = 1.93$ (the g_y band of the [2Fe-2S] clusters) was detected in control cells containing only the expression vector. Microwave power saturation at the g_y band verified the identity of the EPR signal from SoxR-overproducing cells, in that parallel curves were obtained for the [2Fe-2S] clusters in purified SoxR and *in vivo* (Fig. 1B).

As estimated from the amplitude of the EPR g_y band (24), there was $\approx 18 \mu\text{M}$ reduced SoxR [2Fe-2S] clusters in a concentrated sample of the SoxR-overproducing cells. Quantification of the 17-kDa SoxR monomer (using SDS/PAGE and Coomassie staining) yielded an estimate of $\approx 20 \mu\text{M}$ SoxR monomer in the same sample. Thus, at least 90% of the SoxR protein contained reduced [2Fe-2S] clusters in cells not subjected to oxidative stress. This observation is consistent with a recent independent observation using concentrated cells (20).

Although concentrated cells provided a clear EPR signal of SoxR [2Fe-2S] clusters, the procedure was not amenable to determination of biologically relevant kinetics. An experiment was therefore designed to capture the redox state of SoxR [2Fe-2S] clusters in cells by instantly freezing the samples. A sample taken directly from a culture of SoxR-overproducing cells revealed an EPR signal equivalent to $0.35 \mu\text{M}$ reduced SoxR [2Fe-2S] clusters, after correction for the background spectrum of nonexpressing cells (Fig. 2, trace e). When the cells were treated with paraquat (PQ) during vigorous aeration, the EPR signal of the reduced SoxR [2Fe-2S] clusters was completely eliminated (Fig. 2, trace f), consistent with oxida-

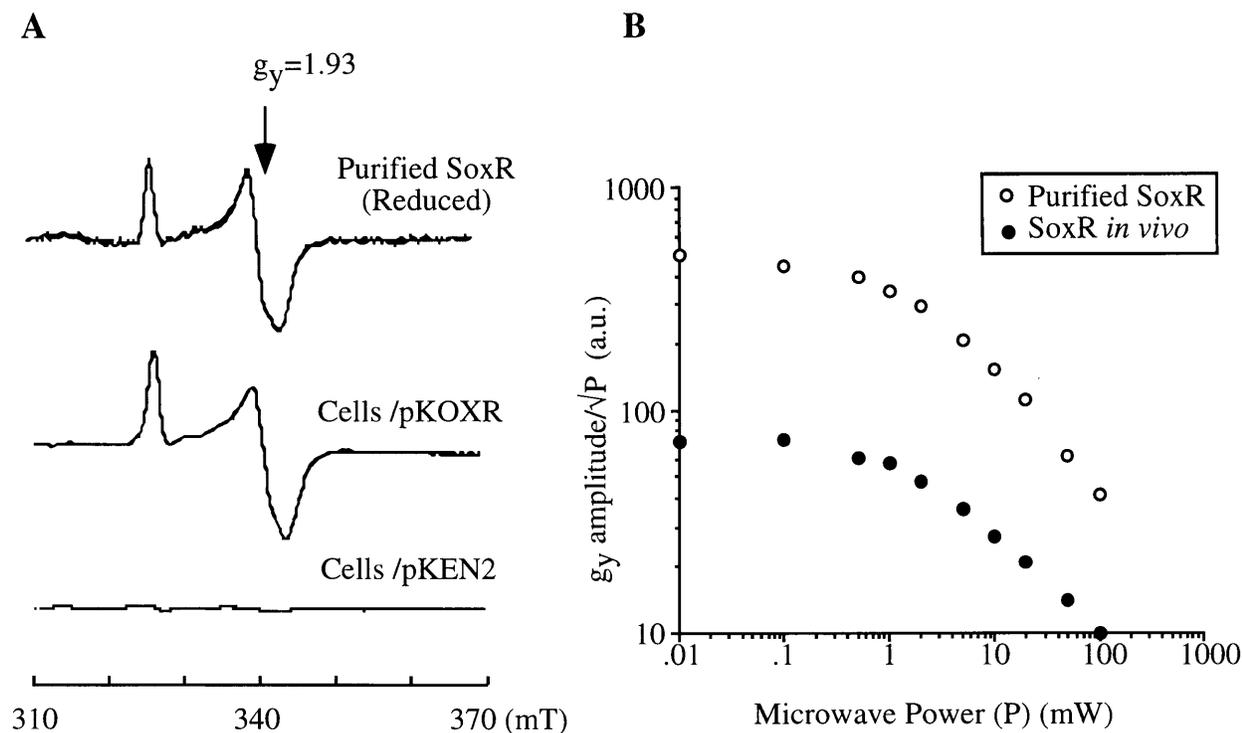


FIG. 1. SoxR [2Fe-2S] clusters are fully reduced *in vivo*. (A) EPR spectra of the SoxR [2Fe-2S] clusters. Top trace, $10 \mu\text{M}$ purified [2Fe-2S] SoxR in 500 mM NaCl and 50 mM Hepes (pH 7.6), reduced with freshly prepared 1 mM sodium dithionite. Middle trace, concentrated SoxR-overproducing cells (strain XA90 containing pKOXR). Bottom trace, concentrated cells containing expression vector only (pKEN2). The printing scales of the cell samples were 2-fold less sensitive than that for purified SoxR. (B) Microwave power saturation of the g_y band of SoxR [2Fe-2S] clusters in purified SoxR and *in vivo*.

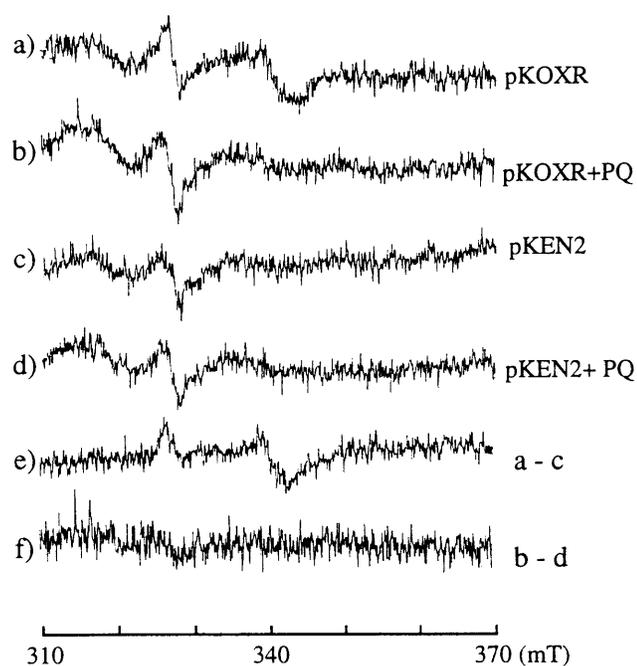


FIG. 2. Oxidation and reduction of SoxR [2Fe-2S] clusters *in vivo*. (Spectra a and b) SoxR-overproducing cells (XA90 with pKOXR) without or with 100 μ M PQ treatment, respectively. (Spectra c and d) Control cells (XA90 with pKEN2) without or with 100 μ M PQ treatment, respectively. Cultures treated with or without PQ were aerated at 275 rpm for 20 min before transfer to EPR tubes and freezing. Ten scans were averaged for each spectrum shown. Trace e is the difference spectrum for trace a with trace c subtracted. From the g_y band amplitude in trace e, the sample contained $\approx 0.35 \mu$ M reduced SoxR [2Fe-2S] clusters. Trace f is the difference spectrum for trace b with trace d subtracted.

tion of the SoxR [2Fe-2S] clusters. Thawing the samples and treatment with 1 mM dithionite did not increase the EPR signal for the cells not exposed to PQ, but did restore the full g_y amplitude for PQ-treated cells (Table 1). Thus, the [2Fe-2S] centers remained intact but EPR silent (i.e., oxidized) after PQ treatment. A full re-reduction of the oxidized SoxR [2Fe-2S] clusters was observed when the oxidative stress was removed by stopping aeration; reapplying aeration when SoxR had been fully re-reduced resulted in its complete reoxidation (data not shown).

The approach described above allowed us to determine the kinetics of SoxR [2Fe-2S] cluster oxidation and reduction *in vivo*. Rapid oxidation (in ≤ 2 min) of SoxR [2Fe-2S] clusters was observed upon aerobic exposure of cells to any of several structurally unrelated redox-cycling compounds that generate intracellular superoxide: PQ, menadione, or phenazine metho-

Table 1. Restoration of reduced SoxR [2Fe-2S] centers by chemical reduction after PQ treatment

Samples	Reduced [2Fe-2S] clusters,* μ M	
	Control	PQ-treated
Directly frozen	0.35 ± 0.04	<0.05
Thawed and dithionite-treated	0.33 ± 0.05	0.32 ± 0.07

Strain XA90 containing pKOXR was treated with PQ as described for Fig. 2 and samples frozen for EPR measurement (Directly frozen). After the EPR measurements, samples were thawed at room temperature, dithionite was added to a final concentration of 1 mM (from a 100 mM stock), and the EPR measurements repeated (Thawed and dithionite-treated). The values shown are the means \pm SD for three independent experiments.

*Calculated from the g_y amplitude.

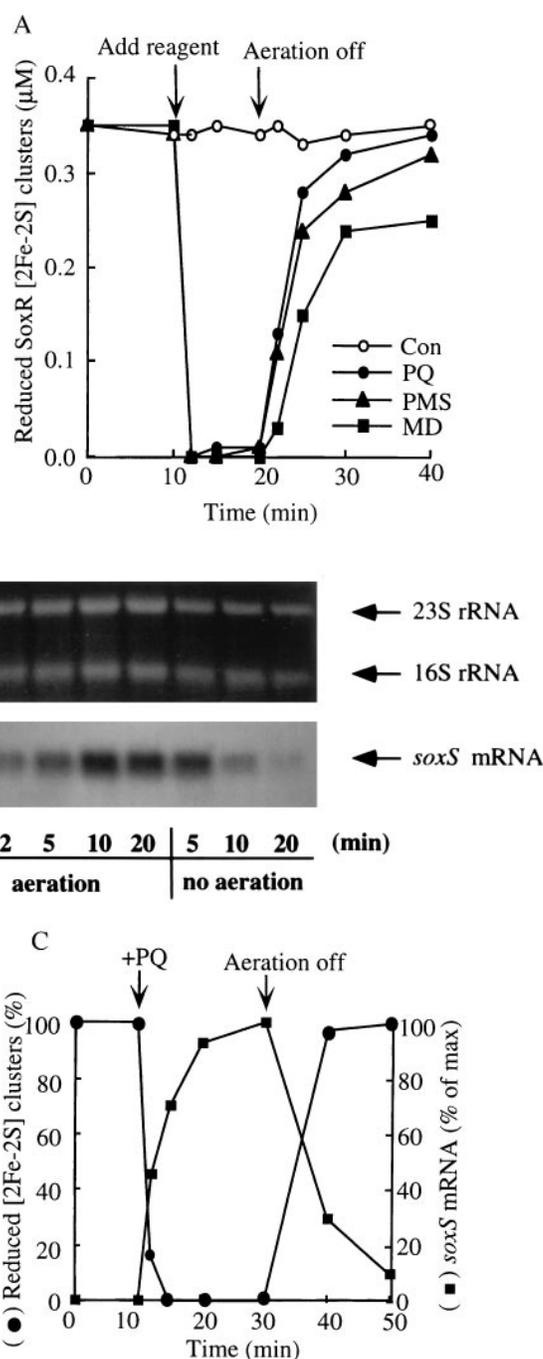


FIG. 3. Kinetics of SoxR activation and inactivation *in vivo*. (A) Kinetics of SoxR [2Fe-2S] oxidation by redox-cycling agents and subsequent re-reduction. SoxR-overproducing cells (XA90 with pKOXR) growing under vigorous aeration were exposed at the indicated time to one of three structurally unrelated compounds: PQ (100 μ M), phenazine methosulfate (PMS, 2 μ M) and menadione (MD, 100 μ M). Aeration was stopped after 10 min as indicated in the figure. Reduced SoxR [2Fe-2S] clusters *in vivo* were quantified using the amplitude of the g_y band in EPR spectra collected as for traces e and f in Fig. 2. Con, cells not treated with redox-cycling agents. (B) Levels of *soxS* mRNA during SoxR activation and shut off. XA90 cells (containing pKOXR) were exposed to 100 μ M PQ as in A, and samples were collected to determine *soxS* mRNA by Northern blot analysis. (Upper) Ethidium staining of the 1% agarose gel to demonstrate constant loading of total RNA. (Lower) Northern blot of *soxS* mRNA. (C) Correlation of the redox state of SoxR [2Fe-2S] clusters and *soxS* mRNA levels. Aliquots of cell culture for EPR measurement at each time point were taken simultaneously with these removed for the *soxS* mRNA determination shown in B. The amount of *soxS* mRNA was quantified as described in *Materials and Methods*.

sulfate (Fig. 3A). Removal of the oxidative stress by stopping aeration led to very rapid re-reduction of the SoxR [2Fe-2S] clusters, such that the protein was mostly reduced within 5 min for PQ- or phenazine methosulfate-treated cells (Fig. 3A). Slightly slower reduction kinetics of SoxR [2Fe-2S] clusters were observed in menadione-treated cells (Fig. 3A), but the level of menadione used in this case was somewhat toxic (inhibitory of cell growth). The results showed that the oxidized SoxR [2Fe-2S] clusters are quickly re-reduced *in vivo* once the oxidative stress is removed. The identities of the cellular reducing activities are unknown (17-20) and are currently under investigation.

Activated SoxR triggers expression of only one known target gene, *soxS* (11, 12). Strains with *soxS'::lacZ* reporter genes have previously been used to monitor *soxS* expression *in vivo* (11). However, the induction of β -galactosidase in PQ-treated cells containing such a *soxS'::lacZ* reporter gene lagged ≈ 10 min behind SoxR oxidation kinetics (data not shown), perhaps due to delays for protein synthesis. We therefore monitored the *in vivo* transcriptional activity of SoxR more directly by following the synthesis of *soxS* mRNA.

Fig. 3B shows that the *soxS* transcript was induced significantly within 2 min of aerobic exposure to PQ, and within 10 min reached a steady state ≈ 100 -fold higher than the level in nonstressed cells. Densitometric quantitation showed that the rapid accumulation of the *soxS* transcript was entirely consistent with the kinetics of SoxR oxidation (Fig. 3C). Moreover, when the oxidative stress was alleviated by stopping aeration, the amount of *soxS* mRNA in the cells declined rapidly, by 20 min nearly reaching the low level observed for nonstressed bacteria (Fig. 3B and C).

The rapid attainment of a plateau level of induced *soxS* message, in cells with continuously activated SoxR, suggested that some other factor might limit accumulation of *soxS* mRNA during oxidative stress. We therefore investigated the stability of *soxS* mRNA as a function of oxidative stress in the wild-type strain GC4468 (Fig. 4A). The RNA polymerase inhibitor rifampicin was used to block new RNA synthesis after induction of *soxS* mRNA. This analysis revealed a significant difference in three independent experiments including that of Fig. 4, with a mean chemical half-life of the *soxS* mRNA $\approx 1.3 \pm 0.4$ min in cells kept under oxidative stress, but $\approx 3.6 \pm 0.6$ min in cells from which the stress was removed. In contrast, the half-life of the SoxR-independent *bla* mRNA (21) was

unchanged by oxidative stress ($\approx 1.2 \pm 0.4$ min), which agrees with previous reports (25). Thus, *soxS* mRNA is specifically destabilized under oxidative stress.

The rapid change in *soxS* mRNA stability upon the removal of oxidative stress suggested control by existing proteins. SoxR was a good candidate for having such a role because of the rapid kinetics with which it is re-reduced (Fig. 3A and C). We tested this possibility by determining *soxS* mRNA stability in the absence of PQ in a strain with a mutant SoxR protein (SoxR101) that is constitutively active even during normal aerobic growth (19, 26). The *soxS* mRNA half-life in this case ($t_{1/2} \approx 1.4$ min) was similar to that for cells with wild-type SoxR activated by oxidative stress (Fig. 4B). This result is consistent with a direct role for SoxR protein in controlling *soxS* mRNA stability. However, it certainly remains possible that another redox-responsive protein, possibly a member of the *soxRS* regulon, governs *soxS* mRNA stability. Because *soxS* transcription is normally tightly dependent on activated SoxR protein, separating out the possible role of SoxR in the stability of *soxS* mRNA will require more sophisticated experiments.

There may be important, strain-dependent differences in the metabolism of redox-cycling agents, which generate intracellular superoxide, and in reducing activities specific for the SoxR [2Fe-2S] centers. For example, Gaudu *et al.* (20) did not observe SoxR oxidation in PQ-treated cells, in contrast to results presented here (Fig. 3A). However, we note that Gaudu *et al.* (20) used a strain of *E. coli* B, which may accumulate PQ intracellularly, unlike the K12 strains we employed in this work (27). Reduced PQ can reduce the SoxR [2Fe-2S] centers *in vitro* (17) and was proposed to do so *in vivo* (20). Other K12 strains may have limited reducing capacity for SoxR: the *in vivo* shut off rate for *soxS* transcription in PQ- or phenazine methosulfate-treated cells was substantially delayed after stopping aeration of strain AB1157 (data not shown), compared with strains XA90 (Fig. 3) and GC4468 (data not shown). The identification of the SoxR reduction mechanism and the enzymes involved in this process may help explain these strain-to-strain variations.

Signal transduction roles for iron-sulfur centers have attracted attention recently (7, 28). For example, assembly and disassembly of the [4Fe-4S] clusters in bacterial Fnr (29) and in mammalian iron-response proteins (30, 31) have been postulated as the molecular switches regulating their activities. SoxR is the first transcription factor with activity that is

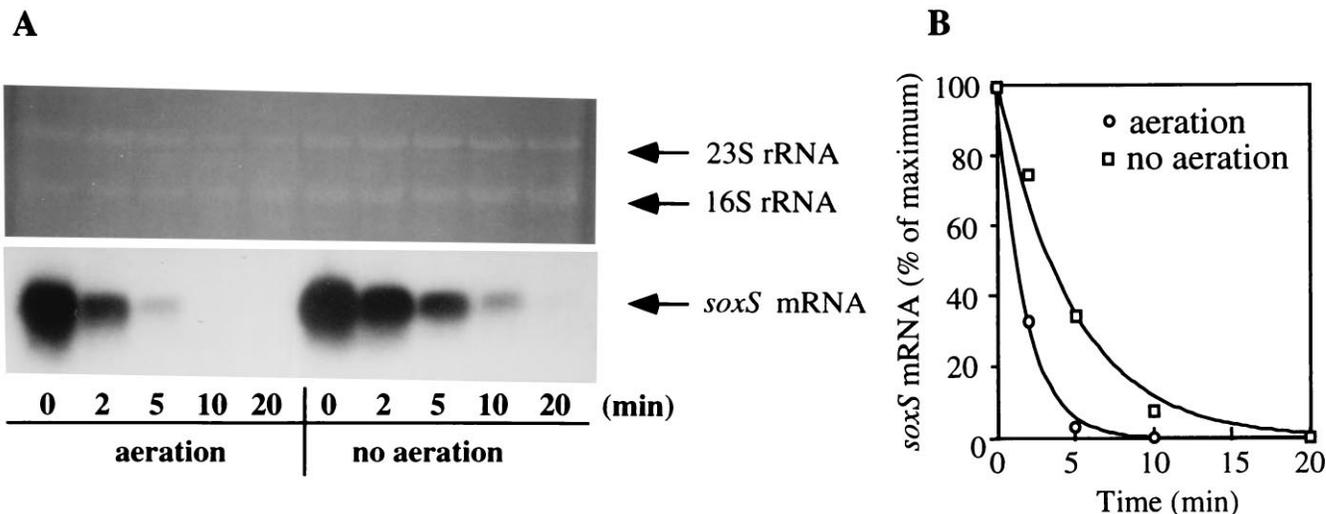


FIG. 4. Half-life of *soxS* mRNA shortened by oxidative stress. (A) Decay of induced *soxS* mRNA. Wild-type *E. coli* (strain GC4468) was used for this experiment. Cells were treated with 100 μ M PQ under vigorous aeration (275 rpm) for 20 min before a final concentration of 150 μ g/ml rifampicin (from a 300 mg/ml stock dissolved in dimethyl sulfoxide) was added to block RNA synthesis. One half of the culture was then subjected to continuing aeration, and aeration stopped for the other half. (B) Quantitation of *soxS* mRNA decay. The curves shown were fitted for exponential decays with a half-life of 1.2 min for the aerated samples and 3.2 min for the nonaerated samples.

demonstrably a direct function of the redox state of its iron-sulfur clusters (17–20). The simple chemistry of iron-sulfur cluster oxidation and reduction facilitates the prompt response of SoxR to the abrupt challenge of oxidative stress. Rapid decay of *soxS* mRNA under oxidative stress may further contribute to the tight control of SoxR redox regulation. It appears likely that such facile, redox-regulated iron-sulfur switches have been exploited in other biological roles.

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