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Biochemistry

SoxR, a [2Fe-2S] transcription factor, is active only in its oxidized form

(*Escherichia coli*/iron-sulfur protein/*soxRS* regulon/superoxide)

PHILIPPE GAUDU AND BERNARD WEISS*

Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109-0602

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ABSTRACT **SoxR protein is known to function both as a sensor and as a transcriptional activator for a superoxide response regulon in *Escherichia coli*. The activity of SoxR was tested by its ability to enable the transcription of its target gene, *soxS*, *in vitro*. The activity of the oxidized form was lost when its [2Fe-2S] clusters were reduced by dithionite under anaerobic conditions, and it was rapidly restored by autooxidation. This result is consistent with the hypothesis that induction of the regulon is effected by the univalent oxidation of the Fe-S centers of SoxR. *In vivo*, this oxidation may be caused by an alteration of the redox balance of electron chain intermediates that normally maintains SoxR in an inactive, reduced state. Oxidized SoxR was about twice as effective as reduced SoxR in protecting the *soxS* operator from endonucleolytic cleavage. However, this difference could not account for a greater than 50-fold difference in their activities and therefore could not support a model in which oxidation activates SoxR by enabling it to bind to DNA. NADPH, ferredoxin, flavodoxin, or ferredoxin (flavodoxin):NADP$^+$ reductase could not reduce SoxR directly *in vitro* at a measurable rate. The midpoint potential for SoxR was measured at −283 mV.**

The superoxide anion radical ($O_2^{\cdot-}$) is a toxic by-product of aerobic metabolism, which is produced mainly by electron leakage from respiratory chain dehydrogenases and other flavoproteins (1). It can also be produced by the autooxidation of foreign compounds that can undergo univalent redox cycling (2), the best known of which is paraquat (methyl viologen). The toxic effects of $O_2^{\cdot-}$ in *Escherichia coli* include DNA damage (3, 4) and damage to the essential iron-sulfur clusters of some [4Fe-4S] enzymes such as aconitase, fumarases A and B of *E. coli*, and dehydratases involved in branched-chain amino acid synthesis (1).

The exposure of *E. coli* to $O_2^{\cdot-}$ generators like paraquat produces a global response that is largely controlled by the *soxR* and *soxS* genes. Induction occurs in a two-step cascade: (*i*) SoxR protein that is activated by oxidative stress induces the transcription of *soxS* and (*ii*) SoxS protein then activates the transcription of other genes (5, 6). The *soxRS* regulon (7–11) contains the gene for Mn^{2+}-superoxide dismutase as well as genes for other products that should reduce $O_2^{\cdot-}$ toxicity, among which are the following: endonuclease IV (a DNA repair enzyme), MicF (which inhibits the synthesis of a porin, thereby blocking the uptake of $O_2^{\cdot-}$ generators), aconitase and an $O_2^{\cdot-}$-insensitive fumarase (which replaces $O_2^{\cdot-}$-damaged enzymes), ferredoxin (flavodoxin):NADP$^+$ oxidoreductase (Fpr; which may be needed for the reactivation of damaged Fe-S centers), and glucose 6-phosphate dehydrogenase (which generates NADPH that is used by Fpr).

SoxR, a ferredoxin-like protein, is a 34-kDa homodimer containing one essential [2Fe-2S] cluster per polypeptide chain (12–14). The presence of these prosthetic groups, which, like $O_2^{\cdot-}$, are capable of univalent oxidation and reduction, immediately suggested that these are the sensor elements that mediate the activation of SoxR. However, little is known about the mechanism of activation. It was proposed (10) that $O_2^{\cdot-}$ does not induce the regulon directly. A key piece of supporting evidence was based on the effects of a *zwf* (glucose 6-phosphate dehydrogenase) mutation. It should decrease the cellular production of NADPH and thus impair the production of $O_2^{\cdot-}$ by paraquat. However, a *zwf* mutation enhanced, rather than reduced, the inducibility of the *soxRS* regulon. Therefore, it was postulated that the inducing signal is a decreased NADPH/NADP$^+$ ratio caused by the consumption of NADPH during the production of $O_2^{\cdot-}$ by redox cycling compounds like paraquat.

The reduced form of SoxR (SoxRred) is readily autooxidized (12, 13). Therefore, aerobically purified SoxR protein ($SoxR_{ox}$) is in an activated, oxidized ($[2Fe\text{-}2S]^{2+}$) state. $SoxR_{ox}$ stimulates the transcription of *soxS* even when the cells from which it was isolated had not been exposed to inducing agents (12, 13). These observations were combined to form the following hypothesis. SoxR is maintained *in vivo* in a reduced, inactive form; it is activated by a shift to its oxidized state when cellular reducing equivalents are depleted by the formation of $O_2^{\cdot-}$ from O_2. It was further suggested (11) that NADPH might reduce SoxR, a ferredoxin-like protein, via reactions linked to ferredoxin or flavodoxin and their reductase (Fpr). Thus, Fpr and glucose 6-phosphate dehydrogenase (which generates NADPH) might provide regulatory feedback when they are induced with the *soxRS* regulon; they might help to restore homeostasis by promoting the reduction and hence deactivation of SoxR.

Clearly, an essential piece of evidence is needed to support the hypothesis that the activation of SoxR is mediated through the oxidation of its Fe-S centers: it must be shown that the reduction of SoxR reversibly inactivates it. This demonstration was elusive because of the instability of the oxidized form, the rapidity with which the reduced compound is autooxidized, and the technical difficulties of maintaining and monitoring anaerobiosis in microscale, multistep reactions. In this study, we overcome these problems and demonstrate that oxidized SoxR is reversibly inactivated by reduction. We also study the binding of the reduced form to DNA. In addition, we measure the midpoint redox potential of SoxR, and we explore a possibility suggested by the work of others (11), that the enzymatic reduction of this ferredoxin-like protein may be mediated directly by bacterial ferredoxin, flavodoxin, or their reductase (Fpr).

MATERIALS AND METHODS

SoxR Protein. SoxR was purified to near homogeneity as previously described (13) except that the following gentler

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Abbreviations: Fpr, ferredoxin (flavodoxin):NADP$^+$ oxidoreductase; $SoxR_{ox}$, oxidized ($[2Fe\text{-}2S]^{2+}$) SoxR; $SoxR_{red}$, reduced ($[2Fe\text{-}2S]^{+}$) SoxR.

*To whom reprint requests should be addressed.

method of cell disruption was used. The thawed cell suspension was incubated in a buffer containing 0.1 M Tris·HCl (pH 7.6), 1 mM dithiothreitol, 0.0025% phenylmethylsulfonyl fluoride, and 0.4 mg/ml egg white lysozyme. After 45 min at 4°C, 45-ml quantities were treated for 5 min with a Heat Systems/Ultrasonics 200-W sonifier (model W225-R) at 40% of maximum power. The yield of purified SoxR was 3.0 to 3.5 mg per gram of wet cells. Its concentration was estimated from the A_{460} of its oxidized form (13), and its molarity is expressed in terms of Fe-S centers (or of polypeptide chains) rather than of dimers.

Enzymes and Cofactors. *E. coli* RNA polymerase-σ^{70} holoenzyme was purchased from Epicentre Technologies (Madison, WI) and phage T7 RNA polymerase from Boehringer Mannheim. Protocatechuate dioxygenase (15) was a gift from D. Ballou (University of Michigan). The following enzymes, which were the generous gifts of those cited, were purified from overproducing strains that contained the cloned *E. coli* genes: ferredoxin (flavodoxin):NADPH oxidoreductase (16) and *E. coli* flavodoxin (17) from D. Hoover (R. Matthews lab, University of Michigan), and *E. coli* ferredoxin (18) from L. Vickery (University of California, Irvine).

DNA and RNA. The DNA template for assays of transcriptional activation by SoxR was a 173-bp PCR product (13) encompassing the *soxS* promoter region. RNA markers for the electrophoresis of transcription products were ^{32}P-labeled runoff transcripts from the phage T7 promoter of plasmid pET11 (19), which were generated *in vitro* with T7 RNA polymerase. A *Bgl*II digest and a *Hae*III digest of pET11 were used to produce 174- and 113-nt RNA products, respectively. The substrate for the DNase protection assays was plasmid pWB33 (5) that was linearized by cleavage with endonuclease *Pvu*II and purified by gel electrophoresis.

Anaerobic Techniques. Anaerobic incubations were performed under an argon stream in 1-ml conical septum vials (Kimble Glass, Vineland, NJ) that were lined with truncated 0.2-ml thin-wall polypropylene PCR tubes. The argon was catalytically deoxygenated by passage through an Oxiclear cylinder (Labclear, Oakland, CA), and then bubbled through an anaerobic solution of 3 mM paraquat/5.2 mM sodium dithionite. The argon was delivered to the reaction and reagent vials through a glass manifold and butyl rubber tubing, and it passed in and out of the vials through hypodermic needles inserted through silicone rubber septa. Gas-tight Hamilton microsyringes were used for liquid transfers. The solution in the tip of a syringe needle, which was assumed to be contaminated with oxygen, was discarded on the inner wall near the top of a vial before a measured amount was added to a reaction mixture. Dithionite-reduced paraquat was present in all anaerobic reaction mixtures, both to remove contaminating O_2 and to serve as an indicator dye for any residual contamination.

Transcriptional Activation Assay. SoxR (3.2 μM) was diluted 10-fold at 4°C in a solution containing 10 mM Tris·HCl buffer (pH 8.0), 150 mM KCl, 150 mM LiCl, 10% glycerol, and 0.05 mg/ml bovine serum albumin, or it was diluted anaerobically in the same buffer plus 3 mM paraquat and 5.2 mM sodium dithionite to obtain the reduced form. SoxR (1 pmol in 3 μl) was added to 18 μl of a solution containing 0.18 pmol of *soxS* DNA template, 67 mM Tris·HCl buffer (pH 8.0), 83 mM KCl, 83 μg/ml bovine serum albumin, and 0.17 mM each of ATP, CTP, and GTP. After 10 min at room temperature, some of the samples were exposed to air for 10 min. Then, 0.3 unit of *E. coli* RNA polymerase was added in 3 μl of a diluent containing 10 mM Tris·HCl buffer (pH 8.0), 50 mM KCl, 30 mM $MgCl_2$, and 0.05 mg/ml bovine serum albumin. After an additional 10 min at room temperature, 3 μl of a heparin sulfate solution (1 mg/ml) was added. The samples were exposed to air, and 3 μl of 80 μM [^{32}P]UTP (3–30 Bq/mmol) was added. After 15 min at 37°C, each reaction was stopped by the addition of 3 μl of 0.1 M Na_3EDTA/50% glycerol and 5 μl of a solution containing 95% formamide, 20 mM Na_3EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol FF. Samples (5 μl) were fractionated by electrophoresis in an 8% polyacrylamide/7.7 M urea gel.

Redox Titration of SoxR. The midpoint redox potential of SoxR was determined by equilibration with the redox dye safranine O (20). The reaction mixture contained SoxR (20 μM), 10.6 μM safranine O (E_o = −289 mV versus a normal hydrogen electrode), 50 mM Mops buffer (pH 7.6), 0.2 M KCl, and 10% glycerol. Reactions were performed under argon in a 1.0-cm light path anaerobic cuvette at 8.0°C. Protocatechuate and protocatechuate dioxygenase were added to 100 μM and 1 μM, respectively, to remove residual O_2. Sodium dithionite solution, standardized with FAD, was added incrementally from a gas-tight syringe. The reduction of SoxR was measured at 415 nm (an isosbestic point for safranine O) using extinction coefficients of 12.46 $mM^{-1}\cdot cm^{-1}$ and 7.52 $mM^{-1}\cdot cm^{-1}$ for the oxidized and reduced forms, respectively. The percent reduction of safranine O was calculated from A_{525} after correction for the absorbance of $SoxR_{ox}$ (ε_{525} = 7.34 $mM^{-1}\cdot cm^{-1}$ and $SoxR_{red}$ ε_{525} = 3.84 $mM^{-1}\cdot cm^{-1}$).

Other Methods. Molecular biological methods that are not specifically described are to be found in refs. 21 and 22.

RESULTS

SoxR Is Reversibly Inactivated by Reduction. The activities of purified $SoxR_{ox}$ and $SoxR_{red}$ were compared by measuring the SoxR-dependent initiation of *soxS* transcription. $SoxR_{ox}$ and $SoxR_{red}$ differ in the oxidation states of their [2Fe-2S] clusters, and they may be interconverted by dithionite reduction and autooxidation (12–14). We also found that reduced paraquat will also reversibly reduce the Fe-S centers of SoxR (data not shown). To reduce SoxR for transcription experiments, we used an excess of sodium dithionite and paraquat under anaerobic conditions (see *Materials and Methods*). The excess reductants helped to guard against the reoxidation of SoxR by the chance introduction of small amounts of air. Because reduced paraquat is rapidly autooxidized from an intense blue to a colorless compound, the state of anaerobiosis could be monitored visually in each of the reaction tubes. A one-cycle transcription assay was used to produce homogeneous runoff transcripts that could be detected as discrete bands by electrophoresis. It was performed in three steps: (*i*) binding—SoxR was incubated with *soxS* DNA; (*ii*) initiation—*E. coli* RNA polymerase-σ^{70} holoenzyme was added in the presence of only three nucleoside triphosphates; and (*iii*) extension—after heparin was added to prevent transcriptional reinitiation, radiolabeled UTP was added to enable completion of the transcripts. The activities of $SoxR_{ox}$ and $SoxR_{red}$ were compared (Fig. 1). $SoxR_{ox}$ was required for the transcription of *soxS* (lane 2 versus lane 1). In its presence, a strong band of mRNA was produced at the expected length of runoff transcripts initiated from the *soxS* promoter (107 nt). This transcription was blocked if SoxR was reduced either before (lane 3) or after (lane 4) the binding step. The inactivation could be reversed by aeration (lanes 5 and 6), a treatment that produces rapid reoxidation of the Fe-S centers of SoxR (12, 13).

In addition to showing mRNA originating from the *soxS* promoter, the overexposed autoradiogram of Fig. 1 reveals faint bands at positions expected for transcripts beginning at one end of the DNA template and extending for its full length (173 nt). These bands provided an internal control. They appeared in all of the lanes, indicating that the activity of RNA polymerase was not significantly affected by the treatments. Therefore, the effects of the redox treatments on the expression of *soxS* mRNA must be attributed their effects on the activity of SoxR rather than on that of RNA polymerase.

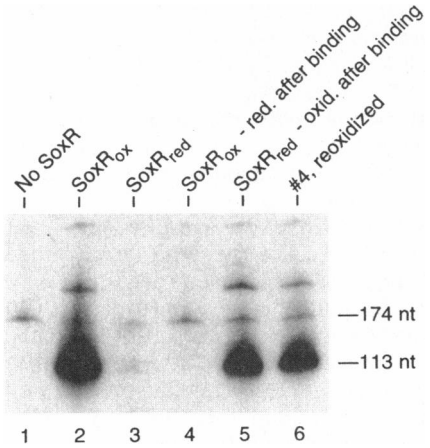

FIG. 1. SoxR$_{ox}$ is reversibly inactivated by reduction. The activity of SoxR was measured by its ability to enhance the transcription *in vitro* of DNA containing the *soxS* promoter as described. An autoradiogram is shown of the electrophoretically separated ^{32}P-labeled RNA products. Except as noted, reactions were under anaerobic conditions. Lanes: 1, no SoxR; 2, SoxR$_{ox}$; 3, SoxR$_{red}$; 4, SoxR$_{ox}$ was reduced after incubation with template and before the addition of RNA polymerase; 5 and 6, same as lanes 3 and 4, respectively, except that SoxR$_{red}$ was reoxidized (aerated) before the addition of RNA polymerase. Also shown are the relative positions of 174- and 113-nt RNA markers, as determined in a separate experiment. red., Reduced. oxid., oxidized.

Operator-Site Binding of SoxR$_{ox}$ and SoxR$_{red}$. We then tested the hypothesis that the inactivity of SoxR$_{red}$ might be due to an inability to bind to the *soxS* operator. In the experiments of Fig. 1, it made no difference if SoxR were reduced either before or after its incubation with DNA (lane 3 versus lane 4), suggesting that the inactivity of SoxR$_{red}$ could not be caused solely by a defect in DNA binding. However, it remained possible that bound SoxR$_{ox}$ might be quickly released upon reduction. To study the binding reaction more directly, we used a DNase protection assay. DNA footprinting experiments (14) have shown that SoxR$_{ox}$ protects the region between the −10 and −35 hexamers of the *soxS* promoter. This operator region contains an 18-bp palindrome at the center of which is a cleavage site for endonuclease *Hpa*I (Fig. 2*A*). Plasmid pWB33 (5), which contains most of the *soxRS* region, has no other *Hpa*I site. Therefore, to determine the relative affinities of SoxR$_{ox}$ and SoxR$_{red}$ for the *soxS* operator, we tested the ability of each to protect pWB33 DNA from cleavage by *Hpa*I (Fig. 2*B*). The reaction conditions were similar to those of the transcription assay. Endonuclease *Pst*I, which cleaves the *bla* gene of the vector DNA, was used as a control for the specificity of binding. The 5.2-kb plasmid DNA was first linearized by digestion with endonuclease *Pvu*II. Subsequent digestions by endonucleases *Hpa*I and *Pst*I in the absence of SoxR produced distinctive patterns for each enzyme and for the mixture (Fig. 2*B*, lanes 1–4). To test for possible inhibitors in the *SoxR* preparation, a protein-free ultrafiltrate was prepared with a Centricon-30 filter (Amicon). It produced only slight inhibition (6%) of *Hpa*I (lane 5). Both SoxR$_{ox}$ and SoxR$_{red}$ protected the DNA against *Hpa*I (Fig. 2*B*, lanes 5–9); in their presence, at least some of the 5.2-kb DNA substrate remained uncleaved. SoxR$_{ox}$ appeared to be about twice as effective as SoxR$_{red}$; the protection afforded by 0.1 μM SoxR$_{ox}$ was about equivalent to that of 0.2 μM SoxR$_{red}$ (lane 8 versus lane 7). In a separate control experiment (results not shown), a dithionite/paraquat mixture, in the amount that would have been introduced with SoxR$_{red}$, did not noticeably inhibit endonuclease *Hpa*I.

The DNase protection observed in Fig. 2*B* occurred through specific rather than random binding of SoxR to the DNA. SoxR protects a region of no more than 36 bp (14), and the reactions

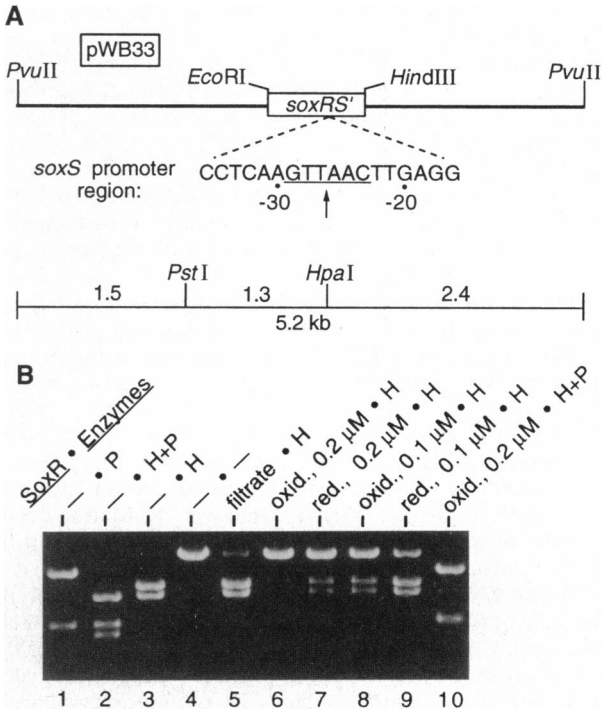

FIG. 2. Binding of SoxR$_{ox}$ and SoxR$_{red}$ to the *soxS* promoter. (*A*) Linearized map of plasmid pWB33 (pBR322::*soxRS'*) (5), the substrate for the DNase protection studies, showing the cleavage sites and site distances for the restriction enzymes used in *B*. (*B*) Nuclease protection assays. *Pvu*II-cleaved pWB33 DNA was incubated with no SoxR, with a protein-free ultrafiltrate of the SoxR preparation, with oxidized (oxid.) SoxR, or anaerobically with reduced (red.) SoxR. The reaction mixtures (20 μl) contained 40 mM Tris·HCl buffer (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, 10 nM pWB33 DNA, and either 0, 1, or 2 μl of a 2-μM SoxR preparation or 2 μl of an ultrafiltrate of 2 μM SoxR$_{ox}$. After 10 min at room temperature, 10 units of endonuclease *Hpa*I and 20 units of endonuclease *Pst*I were added to the indicated reactions. After an additional 40 min, the reactions were stopped by the addition of 2 μl of a solution containing 0.25 M Na_3EDTA, 0.1% SDS, 25% glycerol, and 0.125% bromphenol blue. Products were detected by their fluorescence after electrophoresis in a 1% agarose gel containing EtdBr. red., Reduced; oxid., oxidized; H, *Hpa*I; P, *Pst*I.

contained at most 10 molecules of SoxR dimer per 5.2-kb DNA chain. Therefore, there was not enough SoxR to saturate more than 7% of the *Hpa*I sites by nonspecific binding. The specificity of the assay was confirmed by adding *Pst*I and *Hpa*I DNases together to the SoxR$_{ox}$/DNA mixture; the digestion pattern (lane 10) was that expected for *Pst*I alone (lane 1).

We conclude that both SoxR$_{ox}$ and SoxR$_{red}$ can bind to the *soxS* operator. An approximately twofold difference in the binding affinity of the two forms is not sufficient to account their vast differences as transcriptional activators (Fig. 1 and see *Discussion*).

The Redox Potential of SoxR. SoxR was permitted to equilibrate with the redox indicator dye safranine O while the reducing agent $Na_2S_2O_4$ was gradually added under anaerobic conditions at 8.0°C (Fig. 3). The midpoint redox potential of SoxR was estimated to be −283 ± 4 mV (mean ± SD) versus a normal hydrogen electrode. This value is near the upper end of the range for [2Fe-2S] proteins, which vary from −240 to −460 mV (23). Therefore, relative to these other proteins, SoxR should be readily reduced *in vivo*, given an adequate supply of electrons from a suitable donor.

Fpr Is Not a SoxR Reductase. Both SoxR (13, 14) and *E. coli* ferredoxin (24) have been classified as members of the hydroxylase (adrenodoxin/putidaredoxin) group of [2Fe-2S] proteins, primarily on the basis of EPR spectra that indicated similar conformations of their iron-sulfur clusters. Because of

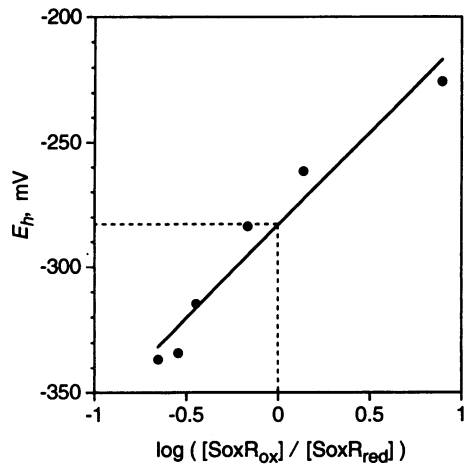

FIG. 3. Determination of the midpoint redox potential of SoxR by spectrophotometric titration. $Na_2S_2O_4$ was added incrementally to an anaerobic cuvette containing SoxR and safranine O as described. At each point, the concentrations of the oxidized forms of both SoxR and safranine O were measured from their absorbances and used to calculate E_h, the redox potential of the system relative to that of a normal hydrogen electrode (20).

this similarity and because Fpr is a product of the *soxRS* regulon, we tested the hypothesis that SoxR may be a substrate for this ferredoxin reductase. SoxR, Fpr, and NADPH were incubated in an anaerobic cuvette at 10°C to avoid the denaturation of SoxR that occurs in concentrated solutions at higher temperatures. The reaction mixture contained 22 μM SoxR, 100 μM NADPH, 10 mM dithiothreitol, 20 mM Mops buffer (pH 7.6), 0.5 M KCl, and 10% glycerol in a total volume of 0.5 ml. At zero time, 1.3 μg of Fpr were added anaerobically. The oxidation of NADPH and the reduction of the Fe-S centers of SoxR were monitored by periodic measurements of A_{340} and of A_{460}, respectively. During 100 min, there was no significant reduction of SoxR (<5%). To prove that the Fpr was active and to provide a point of comparison with the lack of SoxR-driven reduction of NADPH, the cuvette was then opened to the air, and paraquat was added to a final concentration of 4 mM. Fpr, which catalyzes the reduction of paraquat by NADPH (11), caused the NADPH to be completely consumed within 3 min. The results indicate that Fpr does not catalyze the reduction of SoxR at a significant rate.

***E. coli* Ferredoxin and Flavodoxin do Not Reduce SoxR Effectively.** Because of the low redox potentials of ferredoxin (E_o = −380 mV) and flavodoxin ($E_o \doteq$ −450 mV) (25) and because their reduction is catalyzed by Fpr, a member of the *soxRS* regulon, we examined the possibility that they might serve as direct electron donors for SoxR. *E. coli* ferredoxin (1 μM) and *E. coli* flavodoxin (1 μM) were each incubated in an anaerobic reaction mixture containing NADPH, Fpr (1.8 μg/ml), and SoxR under the conditions of the previous experiment. The object was to see if the reduced ferredoxin or flavodoxin produced by the Fpr-catalyzed reaction would in turn reduce SoxR. However, there was no significant reduction of SoxR ($\Delta A_{460} < 5\%$) during 1 h.

Because Fpr is a relatively poor catalyst (26), the reduction of SoxR may have been limited by an inadequate formation of reduced ferredoxin or flavodoxin. Accordingly, we estimated the minimum rate of ferredoxin reduction under our reaction conditions by using cytochrome *c* as an electron acceptor in place of SoxR. NADPH, Fpr, and cytochrome *c* (50 μM) were incubated either with or without ferredoxin. The rate of ferredoxin-enhanced reduction of cytochrome *c* (and hence the rate of ferredoxin reduction) was at least 200 times that of SoxR. Therefore, the reduction of SoxR was not limited by the rate of ferredoxin reduction, and compared with cytochrome *c*, SoxR is a poor electron acceptor for reduced ferredoxin. Although we did not measure the rate of reduction of flavodoxin by Fpr and NADPH, it is reportedly similar to that of ferredoxin (26). Therefore it, too, should not have been limiting in our reactions.

DISCUSSION

$O_2^{\cdot-}$ Is a Univalent Redox Reactant. Because [2Fe-2S] centers mediate one-electron transfers, their presence in SoxR immediately suggested that they are the sensor elements for the *soxRS* superoxide response regulon and that their oxidation level is what regulates the activity. However, they could merely have been structural components that maintain the protein in an active conformation regardless of their redox state. An example of such a protein is aconitase, which is enzymatically active in either its $[4Fe\text{-}4S]^{2+}$ or $[4Fe\text{-}4S]^{+}$ state but not in its [3Fe-4S] forms (27). Our finding that the activity of SoxR depends on the oxidation state of its Fe-S clusters favors the hypothesis that these are indeed sensor elements.

SoxR that has been purified aerobically from cells that were not induced by paraquat or similar agents is in an oxidized form (12, 13), and it is as active as SoxR from paraquat-induced cells (J. Wu and B.W., unpublished data). The oxidized ($[2Fe\text{-}2S]^{+2}$) state of purified SoxR may be easily explained as an artifact of its isolation in the presence of air; chemically reduced $SoxR_{red}$ is autooxidized within a few minutes. Therefore, SoxR may exist primarily as $SoxR_{red}$ in the uninduced cell, and the mechanism of its activation *in vivo*, as well as *in vitro*, may be by oxidation of its Fe-S centers. A crucial piece of evidence consistent with this hypothesis was presented here, namely, that SoxR can be reversibly inactivated by reduction. It has been alternatively suggested that the activity of SoxR may be regulated *in vivo* through a reversible dissociation of the Fe-S clusters of $SoxR_{red}$ (28). However, to explain our results on this basis, the Fe-S complexes would have to dissociate completely within 10 to 20 min, and yet such dissociation was not previously observed during a similar period required for the reductive titration of the Fe-S centers as monitored by EPR (13).

Why is SoxR not constitutively activated by autooxidation in aerobically grown cells? Like many other autooxidizable cellular proteins, its tendency to react with O_2 may be offset by a more efficient pathway for its reduction. According to this model (10, 11), when the reduction of SoxR is impaired by a depletion of reductants like NAD(P)H, the redox balance shifts toward $SoxR_{ox}$, and the regulon is induced. For example, inducers like paraquat consume NADPH during the redox cycling that produces $O_2^{\cdot-}$ as a by-product. Paraquat is reduced univalently by NADPH-linked reductases like Fpr (11). Subsequent autooxidation regenerates the oxidized form of paraquat and produces $O_2^{\cdot-}$. This $O_2^{\cdot-}$ can then oxidize additional NAD(P)H through chains of free radicals as discussed in ref. 10.

Like $SoxR_{ox}$, the SoxR apoprotein (lacking Fe-S centers) is also able to bind to the *soxS* operator and to exist as a dimer, although it is not transcriptionally active (12). What do the Fe-S centers do? A plausible hypothesis was that in the uninduced cell, they exist in a reduced state and deform SoxR so as to block its binding to the *soxS* promoter. Oxidation-sensitive DNA binding is exemplified by the FNR protein of *E. coli*, a transcriptional regulator of a global anaerobic response (29). Our findings contradict this model for SoxR. We found that the reduction of SoxR did not affect its DNA-binding activity as much as it did its transcriptional activity, even though the DNase protection assays (Fig. 2) were performed under conditions similar to those of the initial steps of the transcriptional activation experiments (Fig. 1). The reduction of SoxR decreased its apparent binding to the *soxS* promoter region by no more than half. This difference between $SoxR_{ox}$

Table 1. Constitutive *soxR'* deletion/fusion mutations

soxR allele	SoxR or SoxR' sequence	New amino acids	Molecular weight, $\times 10^{-3}$	Inducibility
soxR$^+$	...CIGCGCLSRSDCPLRNPGDRLGEEGTGARLLEDEQN	0	17.1	+
soxR18	...CIGCGCLSRSDCPLRNPGDRLGEEGTGARLLED	14	18.2	+
soxR107	...CIGCGCLSRSDCPLRNPGDRLGEEGTGAR	148	33.0	C
soxR133	...CIGCGCLSRSDCPLRNPGDR	52	20.9	C
soxR7::cat	...CIGCGCLSRSDCPLRNP	26	17.9	C
soxR4::cat	...CIGCGCLSRSDCPLRNP	49	20.5	C

The fusions are with noncoding or out-of-frame regions, with the exception of *soxR107*, which is a Lac$^+$ *soxR'–'lacZ* fusion. The ends of the SoxR' (truncated SoxR) portion of the protein sequence are shown up to the joint, with the four cysteines of the Fe-S center underlined. The number of new amino acids are those fused to the end of *soxR'*, and the molecular weight is that of the fused protomer. The data are derived from an analysis of mutants that were previously isolated (30). Two additional similar C-terminal deletion/fusions were described (31) in which 19 amino acids were replaced by 8, and 11 were replaced by 15. +, Inducible (wild type); C, constitutively activated.

and $SoxR_{red}$ is insufficient to account for (*i*) the vast difference between their transcriptional activities *in vitro* (Fig. 1 and refs. 12–14), (*ii*) the great increase in *soxS* mRNA after induction (30), and (*iii*) the 47- to 76-fold induction of *soxS–lacZ* fusions (5). Therefore, our results are not consistent with a model that the induction of the *soxRS* regulon is through a redox-activated binding of SoxR to the *soxS* promoter. However, the twofold difference seen in the DNase protection experiments demonstrated that the reduction of the Fe-S centers of SoxR significantly alters its conformation, thereby reducing either its binding constant or its ability, once bound, to protect the soxS operator from endonuclease *Hpa*I. This conformational change is consistent with the postulated role of the Fe-S clusters as redox sensors that control the activity of SoxR.

Two functional domains were predicted from the sequence of SoxR (30). Near its N terminus is a helix-turn-helix (DNA-binding) motif, and near its C terminus is a region postulated to form the Fe-S cluster. This latter region contains the only four cysteines in SoxR, and most [2Fe-2S] clusters are coordinated to four cysteines (23). SoxR may be inactivated *in vivo* by missense (31) or deletion (30) mutations in this region and *in vitro* by extraction of its Fe and S (12); therefore, the Fe-S centers are essential for the activity of SoxR. The Fe-S centers are probably also important for the regulation of this activity because mutations in their proximity produce a regulon-constitutive phenotype. Thus, a variety of mutations affecting the 24 amino acids between the C terminus of the protein and the 4-Cys cluster yield a constitutively activated SoxR derivative. These mutations range from simple missense mutations and a 9-amino acid truncation (31) to more extensive deletions accompanied by fusions to short (31) or to long (Table 1) polypeptides. Therefore, the C-terminal region does not contain a structure required for the activation of SoxR, but it must contain a structure that is needed to maintain it in the uninduced state. Our findings raise the possibility that this region affects the reduction of SoxR. For example, the native sequence may contain a recognition site for a specific reductase. However, the explanation may be a little more complex because these mutations do not have an all-or-none effect; all regulon-constitutive *soxR* mutants that have been isolated so far retain some inducibility, amounting to about a doubling of activity after exposure to paraquat (7, 8, 30, 31).

The redox systems that might reduce SoxR *in vivo* are unknown. In experiments with NADPH, *E. coli* flavodoxin, ferredoxin, and Fpr, we found only that none of these compounds could serve directly as efficient electron donors for $SoxR_{ox}$. It remains possible, however, that one or more of them may be involved indirectly, i.e., as intermediates in a chain of reactions that convert $SoxR_{ox}$ to $SoxR_{red}$.

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