Regulation of the soxRS Oxidative Stress Regulon
REVERSIBLE OXIDATION OF THE Fe–S CENTERS OF SoxR IN VIVO∗

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SoxR protein, a transcriptional activator of the soxRS (superoxide response) regulon of Escherichia coli, contains autooxidizable [2Fe–2S] centers that are presumed to serve as redox sensors. In vitro transcription experiments previously demonstrated that only the oxidized form is active. Reduced SoxR was detected in overproducing strains by EPR spectroscopy of suspensions of intact cells. Oxidized Fe–S centers were determined by lysing the cells and treating them with the reducing agent sodium dithionite prior to EPR measurements. In uninduced cells, 90% of the SoxR was in the reduced form. Treatment with the redox cycling agents phenazine methosulfate or plumbagin was accompanied by reversible oxidation of the Fe–S centers. Mutant SoxR derivatives that were constitutively activated existed constitutively in an oxidized state. The results indicate the presence of a cellular pathway for countering the autooxidation of SoxR and confirm the hypothesis that induction of the regulon is mediated by a shift in the redox equilibrium of SoxR rather than by assembly of its Fe–S clusters.

In Escherichia coli, the soxRS regulon, which consists of over 10 genes (reviewed in Refs. 1–3), mediates an oxidative stress response that protects the cell against the superoxide anion radical (O2−), nitric oxide, and redox cycling reagents, such as paraquat (methyl viologen), plumbagin, and PMS.1 The sensor for the regulon is SoxR protein, a transcription factor that contains [2Fe–2S] centers (4, 5) and whose only known target is the soxS operon. Activated SoxR enhances the production of SoxS, a simple transcriptional activator of the AraC family, which in turn, increases the transcription of the other genes of the regulon (4, 5).

Two models have been proposed for the activation of SoxR. The first model states that it is regulated through the reversible disassembly, either partial or complete, of its Fe–S centers, which might be associated with redox reactions (6, 7). Like most [2Fe–2S] proteins, the Fe–S clusters of SoxR can be easily stripped from the protein and replaced (8). This disassembly may be facilitated in vitro by thiols (6, 7). The apoprotein is inactive although it can still bind to DNA (9), whereas the holoprotein can activate the transcription of soxS (9, 10). This model was probably promoted because of early failures to verify the alternative model (6). However, it cannot easily explain why SoxR that was purified from uninduced cells contained intact Fe–S centers, unless one assumes that there is efficient spontaneous reconstitution of the Fe–S centers in cell extracts.

The alternative model states that SoxR is regulated by the univalent oxidation of its Fe–S clusters, which remain intact. SoxR is readily autooxidized, and the protein that is isolated in ambient atmosphere from uninduced cells is transcriptionally active (6, 10). Recently, we demonstrated that protein can be reversibly inactivated in vitro by reduction of its Fe–S centers with dithionite under conditions that should not have resulted in dissociation of the Fe–S centers (3). This finding was consistent with an observation that the regulon’s induction may be sensitive to the redox balance of the cell (11). However, what we can demonstrate in vitro is not necessarily related to what happens in vivo. In this work, we use EPR spectroscopy to detect SoxRred in overproducing cells, and we show that the redox state of its Fe–S centers changes under inducing conditions.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—E. coli strain BW1027, the host for soxR plasmids, was a transductant of strain BL21(DE3) (12) containing the recA1538 mutation (13), a deletion affecting both soxR and soxS. The expression plasmid pET11K-soxR was described previously (10). Plasmids containing truncated soxR alleles were constructed as follows. An HpaI-SmaI segment of the soxRS region (13, 14) was ligated first into the SmaI site in the BamHI-SmaI-BamHI cloning site region of plasmid pHE6 (15), and the resulting new BamHI fragment was subcloned in pET11K to yield pET11K-soxR21. Another plasmid was constructed by subcloning a truncated soxR gene from one of a set of M13 recombinant plasmids containing terminal soxS deletions adjacent to the vector’s EcoRI site (14). A 560-base pair BamHI-EcoRI fragment was used to replace a BamHI-EcoRI segment of pET11K, producing the plasmid pET11K-soxR22. Prior to ligation, the uneven ends of the restriction fragments were filled in with the aid of the Klenow fragment of DNA polymerase I.

Bacterial Growth Conditions—Bacteria were grown in LB broth (16) supplemented with 1% glycerol and 30 mM potassium phosphate buffer, pH 7.6. Kanamycin (50 μg/ml) was added to cultures of plasmid-bearing strains. Cultures of 20 ml were grown in 300-ml culture flasks (Bellco) possessing 14-mm diameter side-arm cuvettes. Aeration was accomplished by shaking at 250 rpm in a gyratory water bath. Growth was monitored with a Klett colorimeter containing a no. 56 filter; 30 Klett units was equivalent to a cell density of about 109 ml−1. To overproduce SoxR for EPR measurements, a culture of cells bearing a pET11K derivative was grown at 37°C to 50 Klett units, IPTG was added to 0.5 mM, and the culture was grown at 15°C for an additional 12 h. When indicated, a regulon inducer such as PMS, plumbagin, or paraquat was added, and incubation was continued for 30 min. Equal portions of the cultures (Klett units × ml = 2400) were then harvested by centrifugation at 9800 × g for 10 min at 4°C and resuspended in 0.3 ml of cold 50 mM MOPS buffer, pH 7.6, containing 0.2 mM LiCl and 0.2 mM KCl (EPR buffer). The suspensions were transferred to EPR tubes and frozen immediately in liquid N2.

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1 The abbreviations used are: PMS, phenazine methosulfate; SoxRred, reduced (2Fe–2S)2+ SoxR; SoxRox, oxidized (2Fe–2S)2+ SoxR; IPTG, isopropyl-β-D-thiogalactopyranoside; MOPS, 3-(N-morpholino)propane-sulfonic acid; PAGE, polyacrylamide gel electrophoresis.
Oxidative Activation of SoxR

FIG. 1. EPR spectra of intact cells. The strains used were a soxRS deletion mutant (BW1038) either lacking or containing the plasmid pET11K-soxR from which SoxR could be overexpressed in the presence of IPTG. All cultures were treated with IPTG and grown for 12 h at 15 °C prior to other treatments. A, plasmid-free cells. B, plasmid-bearing cells. C, plasmid-bearing cells were incubated with the regulon-inducer PMS (15 μM) for 30 min before harvesting. D, a sample of the PMS-treated cells was washed and incubated under argon for 50 min in the presence of spectinomycin. (see "Experimental Procedures").

Recovery from PMS Treatment—After PMS treatment, the cells were washed by centrifugation at 4 °C in 10 ml of 10 ml Tris-HCl buffer, pH 7.6, containing 10% sucrose and resuspended in 0.3 ml of fresh growth medium containing spectinomycin at 0.1 mg/ml. After transfer to an EPR tube and equilibration with oxygen-free argon for 10 min, the sample was incubated for 50 min at 15 °C and then frozen in liquid N_{2}.

Reduction of Cellular SoxR In Vitro—To 0.3 ml of a cell suspension in EPR buffer were added the following: egg white lysozyme to 0.2 mg/ml, bovine pancreatic DNase I to 30 μg/ml, and a solution of phenylmethanesulfonyl fluoride in acetone to 1 mM. The mixture was incubated 1 h at 4 °C. A solution of 1 mM sodium dithionite in 1 mM MOPS buffer, pH 7.6, was prepared anaerobically, and 80 μl were transferred to an EPR tube under argon and mixed with 300 μl of the lysed cell suspension. The tube was then frozen in liquid N_{2}.

EPR Spectroscopy—EPR measurements were made on a Varian Century X-Band EPR spectrometer operating at 9 GHz, equipped with a 100-kHz modulation. EPR spectra were recorded at 25 K using 0.1 millitesla modulation amplitude and a microwave power of 1 milliwatt. For each spectrum the analog output was recorded digitally on the TRACOR/Northern NS-750 signal averager. SoxR concentrations are expressed in terms of equivalents of [2Fe–2S]^{2+} or of polypeptide rather than of the SoxR dimer.

Iron Determinations—Precautions were taken to reduce contaminating metal ions (17). Cell suspensions were prepared in 50% concentrated HCl to release bound iron, and they were analyzed by inductively coupled plasma atomic emission spectrometry at the Department of Geological Sciences at the University of Michigan. EPR buffer, which served as a blank sample, contained <10% as much iron as the suspensions of whole cells.

Protein Analyses—Protein concentrations were measured with the Pierce Coomassie Blue dye reagent with bovine serum albumin as a standard. Concentrations of SoxR protein in whole cells were determined by SDS-PAGE, using Coomassie Blue-stained 12% gels with purified SoxR (10) standards. Densitometry was performed with a charge-coupled device video camera (Color QuickCam, Connectix Corp., San

Detection of Reduced SoxR in Vivo—EPR spectroscopy may be used to analyze the state of overproduced iron-sulfur proteins in intact cells (20). Therefore, we employed this method to monitor the oxidation state of SoxR in vivo. SoxR was overproduced under the control of a phage T7 promoter in a derivative of the expression vector pET-11 (12), which was contained in a host bearing an IPTG-inducible T7 RNA polymerase. We used mild inducing conditions with the goal of producing just enough SoxR to give a clear EPR signal without overwhelming either the cell’s capacity to produce other proteins or its ability to reduce SoxR. Induction with IPTG was performed at 15 °C to favor the production of the soluble, active SoxR holoprotein (10). Because the host cell bore a chromosomal soxRS deletion, its soxRS regulon was not inducible. Therefore, any change in the EPR pattern after induction would have to be attributed to SoxR rather than to any other product of the regulon. The [2Fe–2S]^{2+} clusters in the reduced form of purified SoxR produce a characteristic EPR spectrum that disappears on oxidation (6, 10). Cells that overproduced SoxR generated a strong EPR signal (Fig. 1B), whereas plasmid-free cells (Fig. 1A) did not. The g values (2.00, 1.92, and 1.90) are characteristic of [2Fe–2S] proteins of the hydroxylation class and are similar to those previously obtained for pure SoxR (6, 10). When the overproducing cells were treated with PMS, a redox cycling compound that generates superoxide and induces the regulon (5, 21), the EPR signal (Fig. 1C) was reduced to that of the plasmid-free cells. Therefore, a regulon inducer causes the disappearance of reduced [2Fe–2S] centers, a result that is consistent with their oxidation or their disassembly.

After PMS treatment, the EPR signal was partially recovered (55%) when the cell suspension was washed to remove most of the compound and purged with oxygen-free argon (Fig. 1D). Because this increase in SoxR^{red} occurred in the presence of spectinomycin, an inhibitor of protein synthesis, it does not represent newly synthesized SoxR. Instead, this result indicates the presence of a cellular system for regenerating reduced Fe–S centers in SoxR.

Detection of SoxR^{red}—The concentration of SoxR^{red} in whole cells was determined from the density of unpaired electron spins obtained by EPR measurements. The total concentration

| Treatment | [SoxR^{red}] | Total [SoxR] | Fraction % |
|-----------|--------------|--------------|------------|
| None      | μM           | μM           | %          |
| PMS, 7 μM | 26.7         | 29.4         | 90         |
| PMS, 15 μM| 3.8          | 27           | 14         |

* Strain BW1038(pET11K-soxR) was treated with IPTG and the indicated amount of PMS under conditions similar to those used in Fig. 1, B and C.

** [SoxR^{red}] is the density of unpaired electron spins measured by EPR on a suspension of intact cells. A background value of 2.0 μM (obtained with plasmid-free cells) was subtracted.

* Total SoxR holoprotein ([SoxR^{red} + SoxR^{ox}]) was determined by EPR spectroscopy after treating the cell suspensions with lysozyme and sodium dithionite under anaerobic conditions (see "Experimental Procedures"). A background value of 2.0 μM was subtracted.

Matoe, CA) connected to a MacIntosh Iic computer using the public domain NIH Image program (developed at the U. S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image).

Other Methods—The following methods were as cited: general anaerobic techniques (3), gel electrophoresis and cloning methodology (18), and assays for glucose-6-phosphate dehydrogenase activity (19).

RESULTS

Detection of Reduced SoxR in Vivo—EPR spectroscopy may be used to analyze the state of overproduced iron-sulfur proteins in intact cells (20). Therefore, we employed this method to monitor the oxidation state of SoxR in vivo. SoxR was overproduced under the control of a phage T7 promoter in a derivative of the expression vector pET-11 (12), which was contained in a host bearing an IPTG-inducible T7 RNA polymerase. We used mild inducing conditions with the goal of producing just enough SoxR to give a clear EPR signal without overwhelming either the cell’s capacity to produce other proteins or its ability to reduce SoxR. Induction with IPTG was performed at 15 °C to favor the production of the soluble, active SoxR holoprotein (10). Because the host cell bore a chromosomal soxRS deletion, its soxRS regulon was not inducible. Therefore, any change in the EPR pattern after induction would have to be attributed to SoxR rather than to any other product of the regulon. The [2Fe–2S]^{2+} clusters in the reduced form of purified SoxR produce a characteristic EPR spectrum that disappears on oxidation (6, 10). Cells that overproduced SoxR generated a strong EPR signal (Fig. 1B), whereas plasmid-free cells (Fig. 1A) did not. The g values (2.00, 1.92, and 1.90) are characteristic of [2Fe–2S] proteins of the hydroxylation class and are similar to those previously obtained for pure SoxR^{red} (6, 10). When the overproducing cells were treated with PMS, a redox cycling compound that generates superoxide and induces the regulon (5, 21), the EPR signal (Fig. 1C) was reduced to that of the plasmid-free cells. Therefore, a regulon inducer causes the disappearance of reduced [2Fe–2S] centers, a result that is consistent with their oxidation or their disassembly.

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Detection of SoxR^{red}—The concentration of SoxR^{red} in whole cells was determined from the density of unpaired electron spins obtained by EPR measurements. The total concentration
of Fe–S centers (both oxidized and reduced) was determined after lysing the cells and treating them with the reducing agent sodium dithionite under anaerobic conditions. This treatment should reduce any SoxRox and allow its detection by EPR. In a SoxR overproducer that had not been treated with a regulon inducer, 90% of the SoxR appeared to be in the reduced form (Table I). Treatment of the cells in vivo with PMS lowered the ratio of SoxRred to SoxRox. However, the EPR signal could be recovered fully by dithionite treatment. Therefore, the decrease in the EPR signals accompanying regulon induction was due to the reversible oxidation of the Fe–S centers of SoxR rather than to their dissociation.

We considered the possibility that during cell lysis and dithionite treatment, some of the Fe–S centers may have dissociated, thereby causing an underestimate of total SoxR concentrations and consequently of the derived values for SoxRox in vivo. The validity of the method was ascertained in three ways. The first was by a recovery experiment. When purified SoxR (15 μM) was treated in the same manner as the whole cells, ≥95% of the EPR signal was recovered. The second method involved measuring the concentration of SoxR polypeptide by densitometry after SDS-PAGE, using purified SoxR as a standard. The results were within 5% of the values obtained by EPR spectroscopy. The third method was by iron determination. Overproduction of SoxR was accompanied by an increase in cellular iron. If this newly acquired iron were in SoxR, it could be used as an independent measurement of SoxR holoprotein. In an experiment similar to that in Fig. 1, a suspension of plasmid-free cells had an iron concentration of 65 μM, whereas a suspension of SoxR overproducers at the same density had an iron concentration of 110 μM. The difference (45 μM) corresponded to 23 μM of [2Fe–2S] centers. This result agreed with the value of 22 μM that was found by EPR measurements of the same sample after dithionite treatment. Therefore, in the uninduced state, SoxR protein was nearly fully populated with [2Fe–2S] clusters, most of which were reduced. This finding contradicts the theory that in uninduced cells, the inactive state of SoxR is due to the dissociation of its Fe–S centers.

Other Redox Cycling Agents—Plumbagin and paraquat are also inducers of the soxRS regulon (13, 21). As expected, treatment of the IPTG-induced cells with 15 μM plumbagin led to a disappearance of the EPR signal in vivo or a concentration of SoxRred of less than 1 μM (results not shown). However, 200 μM paraquat appeared to have no effect on the spectrum of cellular SoxRred. A probable explanation is that the high level of paraquat used and the reduced oxygen level during cell harvesting permitted the accumulation of enzymatically reduced paraquat (PQ−), which can reduce SoxR directly (3). Indeed, EPR spectra of the samples from paraquat-treated cells contained a sharp signal (g = 2.003) that was consistent with the PQ− radical (results not shown).

**State of SoxR in Regulon-constitutive Mutants**—The Fe–S center of SoxR is most likely coordinated to four cysteines, the last of which is located 24 amino acids from the carboxyl terminus. Many deletion or substitution mutations of the C terminus result in a regulon-constitutive phenotype, provided that they spare the four cysteines (3, 13, 22). In strains carrying these mutations, SoxR is in an activated state even in the absence of an inducer of the regulon. The model for the oxidative activation of SoxR implies that constitutively activated mutant proteins might be constitutively oxidized. It has been suggested, for example, that the mutations might alter a recognition site for a SoxR reductase (3, 22).

Two truncated soxR genes were cloned in the same expression vector as soxR1. The soxR21 and soxR22 alleles produced SoxR derivatives lacking the terminal 18 and 9 amino acids, respectively. Although the expression of the soxR genes in these plasmids is under the control of the lac repressor, there was some transcription even in the absence of IPTG, as noted previously (12). Therefore, their regulon-constitutive phenotypes were manifested by an elevation of glucose-6-phosphate dehydrogenase activity (Table II), the gene for which (zuef) is a member of the regulon. After induction by IPTG, measurements of reduced and total SoxR (Table II) were performed by EPR as before. The concentration of SoxR protein in the EPR tubes was also measured by densitometry following SDS-PAGE. The mutant alleles, soxR21 and soxR22, overproduced less SoxR protein (16 and 10 μM, respectively) than the wild type (22 to 29 μM). The mutant proteins were previously noted to be highly unstable during purification, which might account for the lower recovery of Fe–S centers (3 and 7 μM, respectively) than of protein. Nevertheless, neither mutant appeared to contain SoxRred, within the limits of detection, all of the SoxR appeared to be in the oxidized form (Table II). This finding, that constitutively activated mutant SoxR proteins are constitutively oxidized, provides further evidence for the oxidative mechanism of activation of the soxRS regulon.

**DISCUSSION**

Iron-sulfur centers, which have been known for many years as prosthetic groups in proteins (23), may serve different functions. They may mediate electron transfer as in the ferredoxins; they may be structural elements, as in E. coli endonuclease III (24); or they may be at the active site of enzymes, as in aconitase. More recently, some [4Fe–4S] centers have been found to be important for the control of gene expression (25–27). The discovery of the ferredoxin-like [2Fe–2S] clusters in the SoxR protein, which controls an oxidative stress regulon, immediately suggested that these groups are the redox sensor for the regulon and that the ability of SoxR to serve as a
transcriptional activator may dependent on their redox state. The first evidence in this regard was that the SoxR approtein was transcriptionally inactive in vitro (9). Subsequently, it was found that whereas SoxRred is active in vitro (6, 10), it can be inactivated by anaerobic reduction with dithionite and then rapidly restored by autooxidation (3). In this work, we obtained parallel findings in vivo. It was also previously demonstrated that the presence (9) or oxidation state (3) of the Fe–S clusters in SoxR does not significantly affect its binding to its only known target site, the soxS operator. Instead, oxidation enables it, once bound, to initiate transcription mediated by the RNA polymerase-α70 holoenzyme.

Work from another laboratory cited preliminary experiments in which only 25 to 50% of the Fe–S centers in SoxR were populated in vivo (7). In the present work, by using gentle treatments, we were able to demonstrate that about 90% was reduced. Moreover, we found that in the presence of regulon inducers, the concentration of SoxRred decreased and that the remainder of SoxR was in an oxidized state, i.e. it could generate an EPR signal after reduction by dithionite. Theoretically, these changes in EPR signal could represent a dissociation of the Fe–S clusters resulting from induction, followed by a spontaneous reassembly after the cells were opened and treated with dithionite. However, in another study (7), the Fe–S centers of purified SoxR that were disrupted by glutathione reportedly did not reassemble anaerobically in the presence of dithionite. Moreover, this explanation would be contrary to the expectations of either model of activation, because only the holoprotein (in its oxidized form) is active in vitro.

Our results suggested that during aerobic growth under noninducing conditions, as much as 10% of cellular SoxR may be in the oxidized form (Table I). This finding is compatible with previous observations of a measurable level of SoxR activity during normal aerobic growth; the soxR::cat mutation, which makes the regulon noninducible, reduced the constitutive expression of soxS gene fusions about 30% (4). Why is the regulon not fully activated by this low level of SoxRox? Part of the answer may be that both forms of SoxR are capable of binding to the soxS promoter and therefore must compete with one another; SoxRred binds no more than twice as strongly as SoxRox (3). There is also evidence of such competition in vivo; a soxR− allele, when contained on a multicopy plasmid, is dominant over a chromosomal regulon-constitutive soxR mutation (4). The work presented here showed that the products of the competing wild type and mutant alleles are primarily in the reduced and oxidized forms, respectively. This competition would make the regulon responsive to the redox equilibrium of SoxR rather than just to minute amounts of the oxidized form. In addition, we should note that SoxR is a homodimer containing one [2Fe–2S] cluster per polypeptide chain, and we do not know if oxidation of the Fe–S centers in the same dimer occurs cooperatively nor if the oxidation of both centers is needed for activation. If only the latter were true, then when 10% of the Fe–S centers are randomly oxidized, only 1% of SoxR may activated. A hurdle for activation may also exist at the second step of the regulatory cascade. There appears to be a threshold concentration of SoxS needed for activation of at least one regulon gene (4), which might be explained by the autoregulation of soxS (28).

The soxS oxidative stress regulon was originally referred to as a superoxide response regulon because the first inducers that were found were compounds that generate O2− by redox cycling. They could all be reduced in reactions linked to NADPH, and then undergo autooxidation, thereby transferring an electron to dioxygen to produce O2. The conclusion that O2 was the effector appeared to be confirmed by the finding that a regulon gene could be induced by growing a sodAB mutant in the presence of pure O2 (21), i.e. under conditions in which it would be expected to accumulate O2. However, three important findings suggested that the regulon was not induced directly by O2. (i) The regulon was more easily induced in a zwf (glucose-6-phosphate dehydrogenase) mutant than in a zwf− strain (11). This result was unexpected because the mutant should be defective in the generation of the NADPH needed for O2 production by paraquat. (ii) SoxR was readily autooxidized by O2, and the oxidized form was active (6, 10). (iii) Nitric oxide (or perhaps a more reactive derivative thereof) could induce the regulon under anaerobic conditions (29). Therefore, the following theory was proposed (11, 30). SoxRred is inactive, and its Fe–S centers are normally maintained in that state by reactions linked to NAD(P)H, which counteract its autooxidation. Depletion of available reducing equivalents (e.g. NAD(P)H) produces a shift in the redox balance of SoxR to the oxidized (activated) form. O2 may accomplish this by initiating free radical chain oxidations of NADPH (reviewed in Ref. 30). O2− generators accomplish this by depleting the cell of NADPH through redox cycling.

Although many of the inducing agents are small molecules that should be able to oxidize or reduce SoxR directly, this ability cannot explain their effectiveness as inducers because the midpoint redox potential (E0) of an inducer can either be higher or lower than that of SoxR (E0 = −0.28 V) (3). For example, whereas the value for PMS is +0.08 V, that for paraquat is −0.45 V (31). It is by oxidative cycling, using O2 as a final electron acceptor, that these compounds induce the regulon by depleting the cell of the reducing equivalents needed to keep SoxR in a reduced form in the presence of O2. This is best illustrated by paraquat, which is not a good oxidant for SoxR, because it has a lower redox potential. It is enzymatically reduced in the cell with electrons derived from NADPH (11), and reduced paraquat will directly reduce SoxR (3). Therefore, we must explain its activity as an inducer on the basis of its ability to deplete the cell of NADPH by redox cycling, as suggested previously (11, 30).

The general model for the oxidative regulation of SoxR was supported by our previous finding that in vitro, SoxR could be reversely inactivated by reduction with dithionite under anaerobic conditions (3). The model also postulated that there must be an efficient redox pathway for maintaining the autooxidizable protein in a reduced form during aerobic growth (11, 30). The present work demonstrates that pathway. The components of this reductase system must now be identified.

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