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Redox Signal Transduction: Mutations Shifting [2Fe-2S] Centers of the SoxR Sensor-Regulator to the Oxidized Form

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Summary

SoxR is a [2Fe-2S] transcription factor triggered by oxidative stress and activated in vitro by one-electron oxidation or assembly of the iron–sulfur centers. To distinguish which mechanism operates in cells, we studied constitutively active SoxR (SoxR^c) proteins. Three SoxR^c proteins contained [2Fe-2S] centers required for in vitro transcription and, like wild-type SoxR, were inactivated by chemical reduction. However, in vivo spectroscopy showed that even without oxidative stress, the three SoxR^c proteins failed to accumulate with reduced [2Fe-2S] ($\leq$4% compared to $\geq$40% for wild type). One SoxR^c protein had a redox potential 65 mV lower than wild type, consistent with its accumulation in the oxidized (activated) form in vivo. These results link in vitro and in vivo approaches showing novel redox regulation that couples an iron–sulfur oxidation state to promoter activation.

Introduction

Aerobic metabolism provides substantial energetic advantages for aerobic organisms, but they also have to cope with an important side effect of oxygen utilization: the generation of oxygen radicals. E. coli responds to this challenge by activating two multigene defense systems: one against either hydrogen peroxide or S-nitrosothiols (Christman et al., 1985; Hausladen et al., 1996) and another, the *soxRS* regulon, against superoxide ($O_2^{\cdot -}$) or nitric oxide (Hidalgo and Demple, 1996b; Weiss, 1997). Gene activation in response to oxygen radicals has also been observed in yeast (Moradas-Ferreira et al., 1996) and mammalian cells (Schulze-Osthoff and Baeuerle, 1995; Sun and Oberly, 1996), although the underlying molecular mechanisms of those systems are less well understood than are the bacterial systems.

Expression of these oxidative stress regulons is triggered by imbalances in oxygen radical production or elimination, as in the case of activation of the *soxRS* regulon by agents such as paraquat (PQ) (Wu and Weiss, 1992; Nunoshiba et al., 1992; Nunoshiba and Demple, 1993). The *soxRS* regulon is also activated by nitric oxide generated by activated murine macrophages (Nunoshiba et al., 1993, 1995). Expression of the *soxRS* regulon is mobilized in two transcriptional stages. SoxR protein is constitutively expressed and is converted by intracellular redox signal(s) to an active form that stimulates transcription of only one known gene, *soxS*. The SoxS protein is related to the AraC family of transcriptional activators, and it binds and activates all of the regulon promoters (Amábile-Cuevas and Demple, 1991; Wu and Weiss, 1991; Li and Demple, 1994; Jair et al., 1995). The products of the *soxRS* regulon genes mediate an astonishing variety of cellular resistances: to oxidants such as PQ (Tsaneva and Weiss, 1990; Greenberg et al., 1990), to nitric oxide generated by activated macrophages (Nunoshiba et al., 1993, 1995), to a broad array of antibiotics (Chou et al., 1993), and to organic solvents (Nakajima et al., 1995). These features could contribute to clinical problems, such as bacterial virulence or the development of antibiotic resistance, and underscore the importance of understanding the regulation mechanism of the *soxRS* system.

SoxR is both a sensor of oxidative stress and a transcriptional regulator. The purified protein is a homodimer containing two oxidized [2Fe-2S] clusters (Hidalgo et al., 1995; Wu et al., 1995), apparently one in each protein monomer, with each center anchored by a cluster of four cysteine residues near the SoxR carboxyl terminus (Bradley et al., submitted). Although removal of the Fe from SoxR eliminates transcriptional activity, the apoprotein is still a homodimer that binds DNA with unchanged affinity (Hidalgo and Demple, 1994; Hidalgo et al., 1995). The key question of long standing has been whether the resting (inactive) state of SoxR in vivo is the apoprotein or a form in which the [2Fe-2S] clusters are reduced (Hidalgo and Demple, 1994). SoxR in vitro can be reversibly inactivated by exposure to thiols such as 2-mercaptoethanol or glutathione (Hidalgo and Demple, 1994; Ding and Demple, 1996) and reactivated by reassembly of the [2Fe-2S] clusters (Hidalgo and Demple, 1996a). Recent in vitro experiments (Gaudu and Weiss, 1996; Ding et al., 1996) show that reduction of SoxR reversibly inactivates the protein as a transcription factor, suggesting that oxidation activates SoxR in the cell. However, in vivo probes of the mechanism that activates SoxR in response to oxidative stress have been lacking. We chose to address this issue by analyzing the properties of constitutively active mutant forms of SoxR (SoxR^c proteins). They were initially isolated by their elevated resistance to the superoxide-generating agent menadione (Greenberg et al., 1990). Subsequent studies revealed that all of the constitutive mutations were located in the *soxR* gene (Nunoshiba and Demple, 1994). Many constitutive mutations produce single amino acid substitutions, small deletions, or protein fusions to the carboxyl terminus of SoxR (Amábile-Cuevas and Demple, 1991; Wu and Weiss, 1991; Nunoshiba and Demple, 1994). In all of these cases, *soxS* expression was strongly increased during aerobic growth and could be further increased by PQ treatment (Nunoshiba and Demple, 1994). The key question was whether the constitutive mutations lock the SoxR^c proteins in an active conformation or whether the mutant proteins are more sensitive to an activation signal present during normal growth, such as oxygen.

Here we describe the characterization of three SoxR^c proteins with alterations in different parts of the polypeptide. We demonstrate in vivo and in vitro that they contain [2Fe-2S] centers that are required for transcriptional activation in the oxidized form but that fail to remain in the reduced, inactive form. These data support the

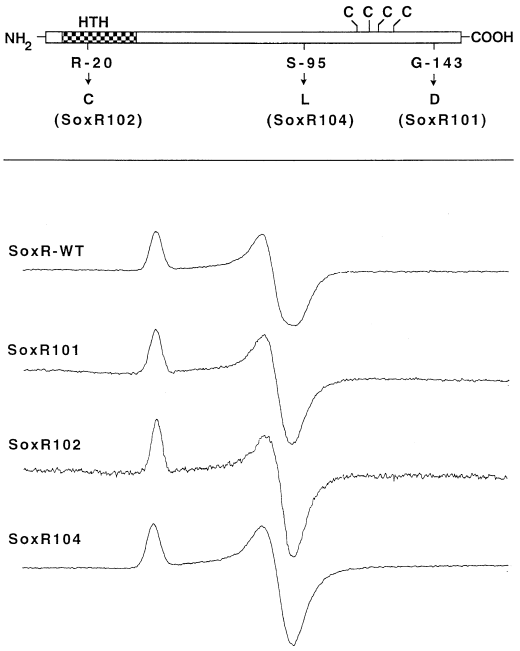


Figure 1. SoxR^c Proteins Contain [2Fe-2S] Centers

(Top) Mutations in SoxR^c proteins. The amino (NH_2)– and carboxy (COOH)—terminal domains are indicated. The checkered area represents the DNA-binding helix-turn-helix (HTH) motif. The relative positions of the four cysteine residues are indicated.

(Bottom) EPR analysis of purified wild-type (WT) and SoxR^c proteins. The protein samples were reduced with 1–7 mM dithionite and subjected to EPR analysis. Four hundred–microliter samples were analyzed at 20°K, microwave frequency of 9.42 GHz, modulation frequency of 100 kHz, microwave power of 1 mW, modulation amplitude of 1.29 mT, time constant of 40.96 ms, magnetic field of 310– 370 mT. SoxR-WT, SoxR101, and SoxR104 were present at ~15 μM (printing scale, 13); the SoxR102 concentration was ~8 μM (printing scale, 12).

hypothesis that oxidation of the SoxR [2Fe-2S] clusters is the mechanism both for sensing oxidative stress and for activating the SoxR protein as a transcription factor.

Results

SoxR^c Proteins Require Oxidized [2Fe-2S] Centers for In Vitro Activity

Many SoxR^c mutations cause deletions or alterations of the SoxR carboxy-terminal region just downstream of the cysteine cluster that anchors the [2Fe-2S] centers to the protein (Nunoshiba and Demple, 1994; Bradley et al., submitted). The *soxR101* mutation changes glycine-143 to aspartic acid and is representative of this class (Figure 1). Two other constitutive alleles encode single amino-acid substitutions in other regions of SoxR (Figure 1): the *soxR102* mutation converts arginine-20 to cysteine in the helix-turn-helix motif, while *soxR104* changes serine-95 to leucine in the center of the polypeptide (Nunoshiba and Demple, 1994). These three constitutive alleles were subcloned into an expression vector and purified. The SoxR^c mutant proteins had chromatographic characteristics indistinguishable from wild-type SoxR protein, and the isolated proteins displayed visible absorption spectra like that of wild-type SoxR (Hidalgo and Demple, 1994). The absorption spectrum is typical of [2Fe-2S] centers, the presence of which in the mutant proteins was confirmed by electron paramagnetic resonance (EPR) spectroscopy. The air-oxidized samples obtained during purification were EPR-silent (data not shown), as expected (Hidalgo et al., 1995; Wu et al., 1995). The dithionite-reduced SoxR^c proteins had clear EPR resonances but with a difference compared to wild-type SoxR: the slightly nonaxial signal detected for SoxR (Hidalgo et al., 1995) had been transformed in the SoxR^c proteins into a near-perfect axial signal, with only two g values (at 2.01 and 1.93) instead of three (Figure 1). This difference suggested that the environment of the [2Fe-2S] centers might be altered in the three SoxR^c proteins.

Although they do not significantly affect DNA binding by SoxR, the [2Fe-2S] centers are essential for full transcriptional activity both in vitro (Hidalgo and Demple, 1994) and in vivo (Bradley et al., submitted). As shown in Figure 2A, the three SoxR^c proteins bound the *soxS* promoter with affinities similar to that of wild-type SoxR. The Fe-containing SoxR^c proteins displayed the same ability as wild-type Fe-SoxR to activate transcription of the *soxS* gene in vitro (Figure 2B). As we might expect (Hidalgo and Demple, 1994; Ding and Demple, 1996), treatment of either wild-type SoxR or SoxR^c proteins with 1 mM 2-mercaptoethanol for 30 min at 37°C disrupted the [2Fe-2S] clusters to generate apoproteins. As for wild-type SoxR, the apo-SoxR^c proteins were unable to activate transcription from the *soxS* promoter in vitro (data not shown). Therefore, the [2Fe-2S] centers are required for transcriptional activity in the SoxR^c proteins just as they are in wild-type SoxR (Hidalgo and Demple, 1994; Hidalgo et al., 1995; Ding and Demple, 1996; Hidalgo and Demple, 1996a). Full reduction with dithionite of the [2Fe-2S] clusters of the SoxR^c proteins and in vitro assay of their transcriptional activity showed that only the oxidized forms were able to trigger *soxS* transcription, as is the case for wild-type SoxR (Gaudu and Weiss, 1996; Ding et al., 1996; Figure 2C). Reoxidation of wild-type SoxR, SoxR101, and SoxR104 restored full transcriptional activity, as judged by the ratio of the *soxS* and control *bla* transcripts (Figure 2C). There was poor recovery of activity with SoxR102 upon reoxidation, suggesting that the [2Fe-2S] centers of this protein might be unstable in the reduced state. The ability of one-electron reduction to block the transcriptional activity of the SoxR^c proteins indicates that they are not simply locked into an active conformation. Instead, we considered the possibility that the *soxR^c* mutations might render the proteins prone to oxidation during normal growth.

The High In Vivo Activity of SoxR^c Proteins Is Dependent on Aerobiosis

The various SoxR^c proteins cause different levels of constitutive expression of a *soxS′::lacZ* reporter gene in the absence of inducers such as PQ. We confirmed these differences by transforming EH46, a *soxR*$^-$ strain carrying a single-copy *soxS′::lacZ* fusion in the chromosome, with the control plasmid (*pSE380*) or expression vectors encoding wild-type SoxR (*pSXR*) or the SoxR^c

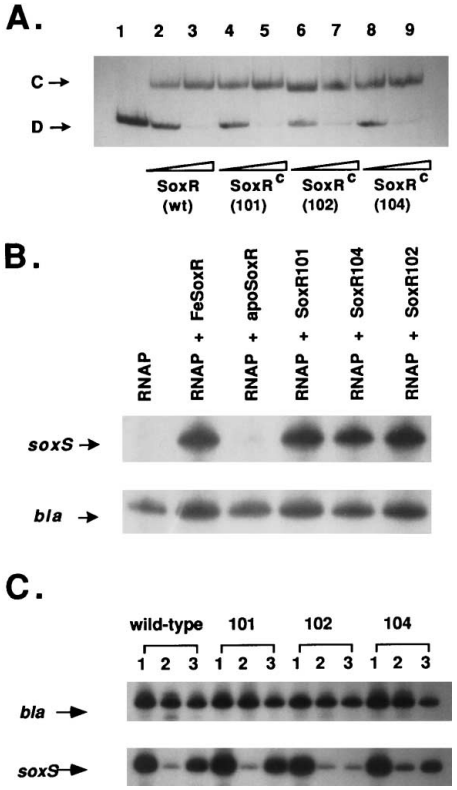


Figure 2. DNA Binding and In Vitro Transcription Activity of Purified SoxR^c Proteins

(A) Specific binding of SoxR and SoxR^c to the *soxS* promoter. The same amount of ^{32}P-labeled *soxS* promoter was incubated under the conditions described in Experimental Procedures without (lane 1) or with 0.1 ng (lanes 2, 4, 6, and 8) or 0.5 ng (lanes 3, 5, 7, and 9) of the indicated SoxR proteins. DNA–protein complexes (C) were separated from unbound DNA (D) by nondenaturing electrophoresis.

(B) In vitro transcription with oxidized SoxR proteins from the wild-type *soxS* promoter. The *soxS*-containing plasmid *pBD100* was incubated with RNA polymerase in the presence or absence of 10 ng of active (FeSoxR) or inactive (apoSoxR) wild-type SoxR or Fe-containing SoxR^c proteins (SoxR101, SoxR102, or SoxR104) in in vitro transcription reactions. The primer extension products for the *soxS* and control (*bla)* transcripts are indicated.

(C) In vitro transcription of *soxS* with dithionite-reduced SoxR proteins. *pBD100* was incubated as described above under anaerobic conditions in the presence of air-oxidized SoxR proteins (lanes 1), dithionite-reduced SoxR proteins (-450 ± 20 mV; lanes 2), or potassium ferricyanide-reoxidized SoxR proteins ($+100 \pm 20$ mV; lanes 3).

proteins (*pTN101*, *pTN102*, or *pTN104*). In this assay, SoxR102 seemed to exert the strongest transcriptional activity, followed closely by SoxR101 and SoxR104 (Figure 3A). As seen previously (Nunoshiba and Demple, 1994), PQ treatment gave at most a small increase in *soxS′::lacZ* expression with the constitutive proteins but yielded a dramatic increase for wild-type SoxR (Figure 3A).

The *soxS* promoter has an unusually long −10/−35 spacing (19 bp) that is overcome by activated SoxR (Hidalgo and Demple, 1994). We have recently engineered mutant *soxS* promoters with reduced −10/−35 spacing that strongly increases SoxR-independent basal expression (Hidalgo and Demple, 1997). For the 18 bp spacer mutants, basal *soxS* expression was unaffected by SoxR, which in contrast exerted a strong repression over the 16 bp and 17 bp mutant promoters (Hidalgo and Demple, 1997). The pattern observed for wild-type SoxR was also seen with the SoxR^c proteins: they had little effect on the 18 bp series (in strains EH56 and EH76; Figures 3B and 3C), and they exerted some repression over the 17 bp series (strain EH66; Figure 3D) and 16 bp series (strain EH86; Figure 3E). The weaker repression exerted by the SoxR^c proteins compared to that of wild-type SoxR was probably due to lower expression of the mutant proteins ($\sim$30% of the amount of wild-type SoxR expressed from the same vector, as determined by immunoblotting; data not shown). Evidently, the binding affinity of SoxR for the *soxS* promoter in vivo is unchanged by the constitutive mutations.

Since SoxR is activated in response to an intracellular redox signal (Wu and Weiss, 1992; Nunoshiba et al., 1992), and the redox state of the wild-type SoxR [2Fe-2S] centers regulates its in vitro transcriptional activity, we determined whether the constitutive activity of the SoxR^c proteins was dependent on growth in oxygen. As a control for expression of the SoxR proteins, under both aerobic and anaerobic conditions, we used the compound diamide, which gives modest induction of *soxS* independent of oxygen (Privalle et al., 1993). As seen in Figure 4A, aerobic *soxS* expression was induced by diamide in strain TN5311-*pSXR* (wild-type SoxR) but was expressed at constitutively high levels in the presence of the SoxR^c proteins. Under anaerobic conditions (Figure 4B), the diamide-mediated induction was still observed for wild-type SoxR. However, the SoxR^c-mediated transcription of *soxS* was significantly diminished and was not increased by diamide (Figure 4B). More stringent anaerobic conditions might lower the SoxR^c-mediated expression of *soxS* still further. To verify that the decrease in *soxS* expression was due to the *soxR^c* alleles themselves and not to other regulatory systems, we determined the contribution of the *arcAB* and *fnr* genes (Lynch and Lin, 1996) to *soxS* expression: mutations in either gene had no detectable effect aerobically or anaerobically (data not shown). Thus, the constitutive activity of the SoxR^c proteins is conditional on cell growth in oxygen.

SoxR^c Proteins with Increased Sensitivity to Oxidation

The oxygen dependence of SoxR^c transcriptional activity suggested that these proteins might be more readily oxidized (activated) than wild-type SoxR. We analyzed the redox state of the SoxR [2Fe-2S] clusters in vivo by overexpressing the wild-type and the SoxR^c proteins in strain TN5311 (carrying a *soxS′::lacZ* fusion) and conducting EPR spectroscopy with concentrated cell pastes (as described in Experimental Procedures) to quantitate the amount of reduced [2Fe-2S] clusters in vivo (Figure 5). Cells expressing wild-type SoxR aerobically contained reduced [2Fe-2S] clusters (determined by EPR) corresponding to at least 40% of the total SoxR protein (determined by immunoblotting; Figure 5). In contrast, only 2–4% of the total SoxR^c protein could be accounted for by reduced [2Fe-2S] clusters (Figure 5).

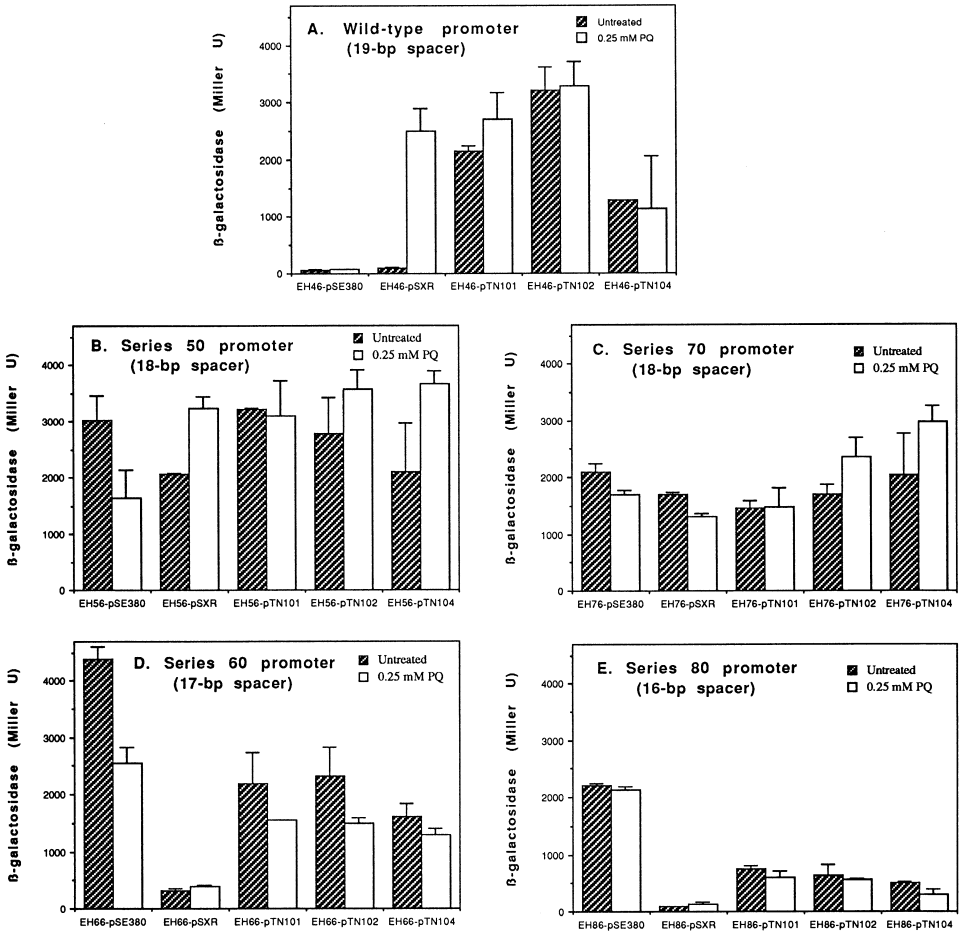


Figure 3. β-Galactosidase Activities of the Wild-Type and Deletion Mutant Promoters in the Presence of Wild-Type or SoxR^c Proteins

Strains containing wild-type (A) or mutant promoters (B–E) fused to *lacZ* and inserted in the chromosome of DJ901 (Δ*soxR*) were transformed with plasmids expressing wild-type SoxR (*pSXR*), the SoxR^c proteins (*pTN101*, *pTN102*, and *pTN104*), or the vector alone (*pSE380*). Cells were grown in the absence (untreated) or presence of 0.25 mM PQ. The spacer size of the different promoters is indicated in each panel for the different mutant series.

The high constitutive activity of the mutant proteins under the assay conditions was confirmed by measurement of *soxS′::lacZ*-directed β-galactosidase activity expressed in the cells before the cell paste was obtained (Figure 5). EPR spectroscopy did not allow us to determine whether the SoxR^c proteins contained oxidized [2Fe-2S] clusters or were present as the apo forms (both are EPR silent). However, a substantial portion of the

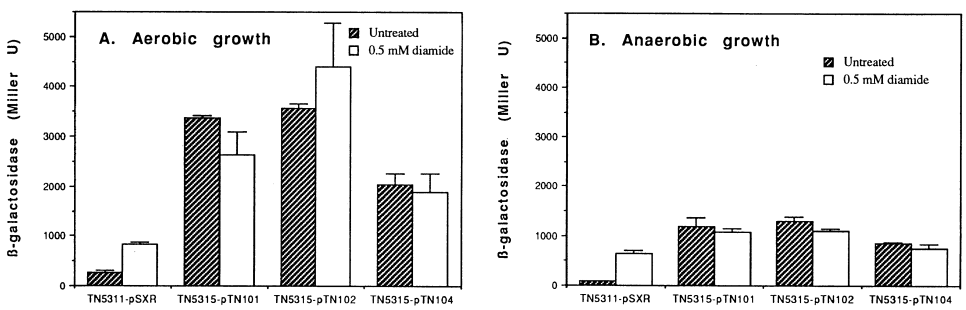
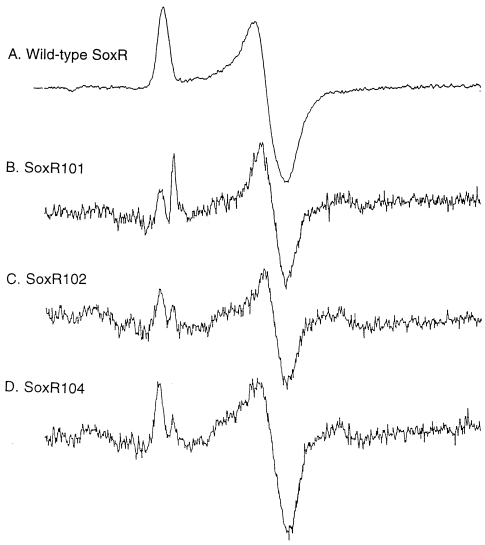


Figure 4. Wild-Type SoxR or SoxR^c-Mediated *soxS* Expression under Aerobic and Anaerobic Conditions

Cells expressing either wild-type SoxR (TN5311-pSXR) or SoxR^c (TN5315-*pTN101*, TN5315-*pTN102*, or TN5315-*pTN104*) proteins were grown under aerobic (A) or anaerobic (B) conditions and under uninduced (untreated) or induced (0.5 mM diamide) conditions, as described in Experimental Procedures. SoxR-mediated *soxS* expression was monitored by measuring β-galactosidase activity of the *soxS′::lacZ* fusion inserted in the chromosomes of both TN5311 and TN5315.



EPR analysis of wild-type and SoxR^C proteins *in vivo*

Strain	ß-gal. activity (U)	[SoxR] (μM)	[red. SoxR] (μM)
A. pKOXR (WT)	199	85	37
B. pTN101K	3625	79	3.0
C. pTN102K	3416	123	2.3
D. pTN104K	2016	103	3.5

Figure 5. In Vivo Quantitation of Reduced [2Fe-2S] Centers

(Top) EPR detection of [2Fe-2S] centers in wild-type and SoxR^c proteins in vivo. Cells expressing wild-type or SoxR^c proteins from the *pKEN2* vector were grown and collected as described in Experimental Procedures. A spectrum for cells carrying only the *pKEN2* vector was substracted electronically before the spectra were printed. The scale for the mutant proteins is 4-fold more sensitive than that for wild-type SoxR, and the spectra for the cell samples with SoxR^c proteins are the sums of three scans to generate sufficient signals for quantitation.

(Bottom) Quantitation of in vivo EPR data, SoxR levels, and in vivo activity. In vivo basal activity of the SoxR proteins was monitored prior to harvest of the cells by measuring β-galactosidase (β-gal.) expressed from a *soxS′::lacZ* fusion inserted in the chromosome. Total SoxR protein in the cell paste was determined by immunoblotting as described in Experimental Procedures; the content of reduced SoxR (red. SoxR) was estimated from the amplitude of the EPR resonances at g_y (Ding and Demple, 1996) normalized to a known preparation of purified SoxR.

SoxR^c proteins in vivo evidently exists with oxidized [2Fe-2S] centers, because neither the apo nor the reduced forms of the SoxR^c proteins are able to trigger *soxS* transcription.

A preponderance of oxidized SoxR^c [2Fe-2S] centers could arise by several mechanisms (Nunoshiba and Demple, 1994), including increased sensitivity to oxidation through a change in the midpoint redox potential of the centers. We addressed this possibility by determining the in vitro midpoint redox potentials of the purified SoxR^c proteins in parallel with wild-type SoxR protein (Figure 6). The observed potential for wild-type SoxR was −285 ± 10 mV, as found previously (Gaudu and Weiss, 1996; Ding et al., 1996), and the redox potentials for the SoxR101 and SoxR102 proteins did not differ significantly from that value. However, the potential for

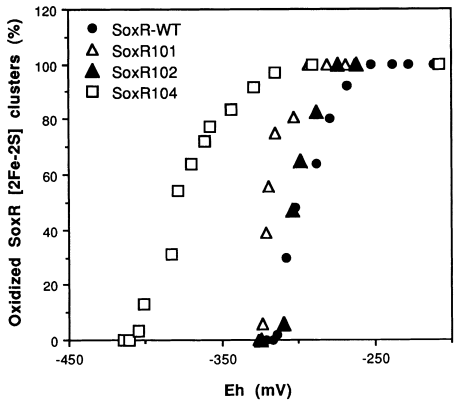


Figure 6. Determination of the Mid-Redox Potential of Wild-Type SoxR and SoxR^c Proteins

Sodium dithionite was gradually added under a continuous argon flow to an anaerobic cuvette containing SoxR and the mediator safranine O, as described in Experimental Procedures. The concentration of reduced and oxidized SoxR, normalized to 0 or 100% for fully reduced or oxidized SoxR, respectively, is plotted against the redox potential measured with a microelectrode. The experiment was repeated one to three times per each protein.

the SoxR104 protein was shifted by −65 mV to −350 ± 10 mV (Figure 6). Such a shift would promote the oxidation of SoxR104 under conditions where the wild-type protein can be maintained in the reduced state.

Discussion

Considerable attention has been focused on the molecular signal transduction mechanisms that activate genetic responses to oxidative stress (Schulze-Osthoff and Baeuerle, 1995; Hentze and Kühn, 1996; Hidalgo and Demple, 1996b; Sun and Oberly, 1996; Moradas-Ferreira et al., 1996; Weiss, 1997). Identifying the sensor proteins that govern these responses has been a paramount goal, and the E. coli SoxR protein has emerged as a paradigm for which well-defined hypotheses can be tested. The experiments reported here have united in vitro and in vivo approaches to demonstrate regulation of SoxR activity by a novel mechanism for a transcription factor: reversible one-electron oxidation (activating) and reduction (inactivating) of the protein [2Fe-2S] centers.

The biochemical properties of the three SoxR^c proteins studied here provide compelling evidence for post-translational redox activation via the iron–sulfur centers (Figure 7). Although the three mutations alter different regions of the SoxR polypeptide, they have a common overall effect: failure of the proteins with reduced [2Fe-2S] clusters to accumulate. The SoxR^c proteins have not lost the ability to respond to changes in the redox state of their iron–sulfur centers and still lose transcriptional activity upon reduction. At least one of the SoxR^c proteins (SoxR104) seems to derive its constitutive activity from a shift in the redox potential of its [2Fe-2S] centers that evidently renders them hypersensitive to oxidation even during normal aerobic growth. It is noteworthy that the change in SoxR104 inserts an additional leucine in

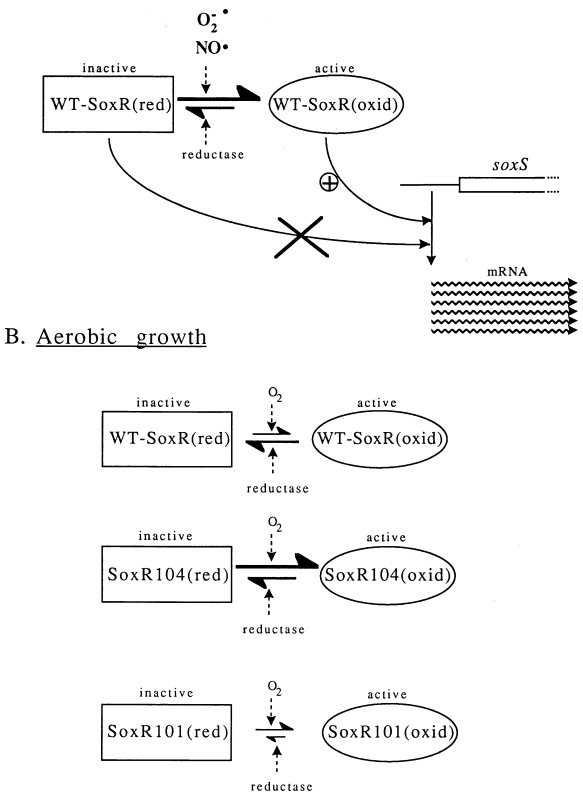


Figure 7. An Integrated Model for SoxR Regulation

(A) During oxidative stress, SoxR activity is positively affected by oxygen radicals to generate the transcriptionally active form with oxidized [2Fe-2S] centers (SoxR(oxid)); this effect is countered by reduction to generate the inactive reduced form (SoxR(red)). Both forms of SoxR bind the *soxS* promoter, but only the interaction of SoxR(oxid) stimulates initiation by RNA polymerase (arrow with plus symbol).

(B) The SoxR^c proteins are activated by aerobic growth. Normally, wild-type SoxR is maintained in the reduced state by the dominance of reduction over oxidation (top). In SoxR104 the balance may be skewed toward oxidation by the negative shift in midpoint redox potential, favoring oxidation (middle). In SoxR101 and perhaps SoxR102, diminished interaction with a reductase could skew the balance by disfavoring reduction (bottom).

a leucine-rich region in the center of the protein (Nunoshiba and Demple, 1994); perhaps this change affects the interaction of the subunits in the dimer in a manner that influences the redox properties of the [2Fe-2S] centers. The other SoxR^c mutations might alter the protein to trap the [2Fe-2S]-oxidized form by other mechanisms. For example, it seems likely that the iron–sulfur centers are maintained in the reduced state by enzymatic reduction. SoxR^c mutations could interfere with a reductase reaction by affecting binding or electron transfer. SoxR101 could fall into this category, because numerous constitutive mutations map to this region of the protein, including in-frame deletions (Nunoshiba and Demple, 1994; Gaudu and Weiss, 1996). The subtle change in the EPR spectra of the SoxR^c proteins could reflect structural alterations that change either the redox potential or the reductase reaction. A possible role for DNA in affecting the redox properties of SoxR should also be considered, since at least one of the constitutive mutations changes the likely DNA binding region of SoxR (SoxR102).

How might wild-type SoxR be activated? Two formal possibilities are evident: direct oxidation of the [2Fe-2S] centers by superoxide or accumulation of the oxidized protein due to diminished reductase activity. These possibilities are not necessarily mutually exclusive but instead may constitute two facets of the overall regulation of SoxR (Figure 7). Oxidation of SoxR could be mediated by oxygen, a possibility that in part would explain the conditional nature of the SoxR^c mutations, that is, the strong dependence on aerobic growth. A critical regulatory role for a reductase would also predict that diminished levels of reduced nicotinamide adenine dinucleotide phosphate (NADPH), usually the ultimate electron source for enzymatic reduction of iron–sulfur centers, could favor the accumulation of oxidized SoxR. In fact, redox-cycling agents such as PQ are potent activators of SoxR and cause the consumption of NADPH (Liochev and Fridovich, 1992; Liochev et al., 1994). Consistent with the prediction that reduction of SoxR may be a regulatory step is the lowered threshold for SoxR activation by PQ in strains deficient in glucose-6-phosphate dehydrogenase (Liochev and Fridovich, 1992), an enzyme that likely contributes to cellular NADPH pools (Fraenkel, 1996). However, changes in reductase activity alone are not likely to explain either the enhanced activation of SoxR by NO· under anaerobic compared to aerobic conditions (Nunoshiba et al., 1993) or the anaerobic induction by the thiol oxidant diamide (Privalle et al., 1993). For those cases, another oxidant must exist, perhaps nitric oxide itself for activation by NO·. The identification of reductase activities specific for SoxR will be an important area of future work.

We noted previously (Nunoshiba and Demple, 1994; Hidalgo and Demple, 1996b) that the powerful constitutive activity exerted by single–amino-acid substitutions in SoxR contrasts with the small effects of single replacements in the homologous MerR protein (Parkhill et al., 1993). In the latter case, mutations must be combined to achieve a similar degree of constitutive activity in the absence of the physiological inducer, Hg^{2+}. The present observations provide a simple explanation for this difference: in the case of SoxR, the protein can be activated by oxidation by normal metabolites ($O_2^{\cdot -}$ or O_2), but for MerR, significant amounts of activating Hg^{2+} are not normally present.

Signal transduction by oxidation of a [2Fe-2S] center constitutes a unique mechanism. Iron–sulfur centers are involved in other examples of redox-responsive genetic regulation, but by changes in the stability of the metal centers. The Fnr protein is a transcription factor with DNA-binding activity dependent on a [4Fe-4S] center, which is destroyed upon exposure to oxygen (Green et al., 1993; Beinert and Kiley, 1996). The activity of the mammalian iron-response protein (IRP) can be triggered by oxidative conditions (H_2O_2 or nitric oxide, for example; Hentze and Kühn, 1996; Roualt and Klausner, 1996) or by changes in the stability of its [4Fe-4S] center rather than the oxidation state per se: only apo-IRP binds its target RNA sites (Hentze and Kühn, 1996; Roualt and Klausner, 1996). Because SoxR activity can also be regulated by assembly and disassembly of the [2Fe-2S]

centers (Ding and Demple, 1996; Hidalgo and Demple, 1996a), the apoprotein may constitute another inactive form in vivo. We note that not all of the wild-type SoxR protein may be accounted for as the reduced [2Fe-2S] form in vivo (Figure 5) and that the EPR-silent apo-SoxR may be present in significant amounts in the cell.

In contrast to Fnr and IRP, the DNA binding affinity of SoxR is not strongly affected by its metal centers (Hidalgo and Demple, 1994) or their oxidation state (Gaudu and Weiss, 1996). Instead, activation of the SoxR transcription complex is an allosteric event in which the structure of the nucleoprotein complex is altered (Hidalgo and Demple, 1994; Hidalgo et al., 1995). The only other known case in which the oxidation state of an iron–sulfur center exerts an allosteric effect is *Azotobacter vinelandii* nitrogenase, in which reduction of an unusual intersubunit [4Fe-4S] center activates a distant ATPase site (Georgiadis et al., 1992). The [2Fe-2S] centers of SoxR appear to be anchored individually within the subunits rather than between them (Bradley et al., submitted; H. D. and B. D., unpublished data) and are quite stable in the oxidized state (Hidalgo and Demple, 1994; Wu et al., 1995). It seems possible that the net positive charges generated by oxidation of both [2Fe-2S] centers in the homodimer could mediate a structural transition in the protein–DNA complex sufficient for activating RNA polymerase. The stability of the SoxR [2Fe-2S] centers in the oxidized state makes them well suited to the signal transduction role we have proposed and suggests that iron–sulfur clusters of this type could be widely used in sensing oxidative stress. Structural studies of SoxR will be of relevance in establishing the mechanistic links between the sensing reaction that oxidizes SoxR and the transduction of gene activation.

Experimental Procedures

Strains and Plasmids

We used for our studies the E. coli K12 strains TN5311, derived from the *soxRS*$^-$ strain TN531 (DJ901 [Δ*soxR*] derivative containing a *soxS′::lacZ* fusion inserted in the chromosome [Nunoshiba and Demple, 1994]) containing F′ *proAB lacI*^{q}ZDM15 Tn*10*, transferred from XL1-blue by conjugation; TN5315, also derived from TN531 but *recA56 srlC300*::Tn*10* (Nunoshiba and Demple, 1994); and XA90 (Nunoshiba et al., 1992). Strain TN5315 was transformed with expression plasmids *pTN101*, *pTN102*, or *pTN104*, derivatives of *pSE380* containing the *soxR101*, *soxR102*, or *soxR104* alleles, respectively (Nunoshiba and Demple, 1994). Strain TN5311 was transformed with *pSXR*, also a *pSE380* derivative containing the wild-type *soxR* allele (Amábile-Cuevas and Demple, 1991; Ding et al., 1997). Strains TN5311 and XA90 were also transformed with high expression plasmids containing the wild-type *soxR* gene (*pKOXR*; Nunoshiba et al., 1992), the constitutive mutant alleles (*pTN101K*, *pTN102K*, or *pTN104K*), or the vector alone, *pKEN2* (Nunoshiba et al., 1992). Other strains used in this study were DJ901 (Δ*soxR*) derivatives containing *soxS′::lacZ* fusions inserted in the chromosome, either the wild-type *soxS* promoter (strain EH46; 19 bp spacer) or *soxS* deletion mutant promoters (strains EH56 and EH76, 18 bp spacer; EH66, 17 bp spacer; or EH86, 16 bp spacer) (Hidalgo and Demple, 1997). Those strains were transformed with the above-described plasmids *pSE380*, *pSXR*, *pTN101*, *pTN102*, or *pTN104*.

Purification of SoxR^c Mutant Proteins

Iron-containing SoxR (Fe-SoxR) and apo-SoxR were obtained from E. coli strain XA90 containing the SoxR expression plasmid *pKOXR* using purification buffers containing 1 mM 2-mercaptoethanol (apo-SoxR) or lacking added thiols (Fe-SoxR), as described previously (Hidalgo and Demple, 1994), but incorporating two modifications: first, we used a cationic exchange phosphocellulose (Whatman P-11) column instead of the previously described heparin–agarose column (Hidalgo and Demple, 1994; Wu et al., 1995); second, we used as purification buffers for both wild-type and mutant proteins a 20 mM MOPS/KOH (pH 7.6), 0.2–0.55 M KCl solution instead of the previously described HEPES/NaCl buffer solution (Hidalgo and Demple, 1994) in order to increase the solubility of SoxR (Wu et al., 1995). The mutant SoxR^c proteins were purified by the same procedure from strains overexpressing the different SoxR proteins (TN5311 carrying *pTN101K*, *pTN102K*, or *pTN104K*). Fractions eluted from phosphocellulose columns (SoxR purity of ≥80%) were used for these studies. To prepare samples for EPR spectroscopy, 8–15 μM solutions of wild-type SoxR or SoxR^c proteins were placed inside 4 mm EPR sample tubes (before or after treatment with 1–7 mM sodium dithionite), frozen in liquid nitrogen, and kept at −80°C until analysis (see below).

DNA–Protein Binding

Plasmid *pEH44*, containing the wild-type *soxS* promoter (Hidalgo and Demple, 1997), was digested with BamHI and EcoRI and its ~148 bp insert isolated after electrophoresis in a 1% agarose gel. The DNA was extracted from the gel slices by a gel-purification procedure (Qiagen). The DNA fragment was labeled and purified from unincorporated nucleotides as described elsewhere (Hidalgo and Demple, 1997). DNA and the indicated amounts of either wild-type SoxR or SoxR^c proteins were incubated in binding reactions and protein–DNA complexes were separated from free DNA by non-denaturing gel electrophoresis (Hidalgo and Demple, 1994).

In Vitro Transcription

The in vitro ability of the different SoxR^c proteins to trigger transcription of the *soxS* gene was analyzed by incubating 10 ng of the different SoxR preparations with the same amount of RNA polymerase and using a plasmid containing the wild-type *soxS* promoter, *pBD100*, as a template (Hidalgo and Demple, 1996a). The *soxS* transcript and a control *bla* transcript were quantified by primer-extension with, respectively, primer *soxS-1* and primer *pBR-1* (Hidalgo and Demple, 1996a). When indicated, purified SoxR proteins were incubated at the concentrations noted above inside an anaerobic cuvette in the presence of the redox mediator safranine O (10 μM) in 100 mM Tris-HCl (pH 7.5) and a redox microelectrode. Dithionite or potassium ferricyanide were slowly added (as described below) to modify the redox potential of the reaction. Forty-microliter samples were then withdrawn at the desired redox potentials and transfered to an argon-degassed vial with the appropriate amount of RNA polymerase. After 3 min of incubation, the in vitro transcription reactions were stopped and processed as described previously (Hidalgo and Demple, 1996a).

Preparation of Cell Suspensions

Strain TN5311 transformed with the expression plasmid *pKEN2* or its derivatives *pKOXR* (containing the wild-type *soxR* gene; Amábile-Cuevas and Demple, 1991), *pTN101K*, *pTN102K*, or *pTN104K* (each carrying one constitutive *soxR* allele) were grown overnight in Luria broth containing ampicillin (100 μg/ml). After a 1:100 dilution from the overnight culture into a fresh 125 ml aliquot of the same medium, incubation proceeded at 37°C with shaking at 225 rpm for 110 min. At that time, 0.5 mM isopropyl-β-D-thiogalactopyranoside was added, and the incubation continued at 37°C with shaking for an additional 120 min. A small culture aliquot was kept for estimation of total β-galactosidase activity (see below). The cell pellet, harvested by centrifugation, was then resuspended in 0.5 ml of 50 mM HEPES-NaOH (pH 7.6), 0.1 M NaCl. Three hundred to 400 μl of the cell paste was then placed inside 4 mm EPR sample tubes, frozen in liquid nitrogen, and kept at −80°C until analysis. The total SoxR concentration in the cells was determined by immunoblot analysis, using previously quantified SoxR as a standard.

Preparation of Antisera

Wild-type E. coli SoxR protein was purified to near homogeneity as described previously (Hidalgo and Demple, 1994). Fractions eluted after affinity chromatography (Hidalgo and Demple, 1994) were

emulsified in complete Freund's adjuvant and injected subcutaneously into two New Zealand black rabbits. Starting 3 weeks after the primary injection, the rabbits received booster injections every 2 weeks with similar SoxR preparations emulsified in incomplete Freund's adjuvant. Polyclonal antisera were extracted periodically starting 4 weeks after the first injection, and SoxR-specific antibodies were purified by affinity chromatography using SoxR-columns generated by coupling purified SoxR protein to HiTrap NHS–activated columns (Pharmacia) according to the conditions recommended by the supplier. Polyclonal serum was applied to the columns and SoxR-specific antibodies eluted according to standard procedures (Harlow and Lane, 1988).

Western Blot Analysis

For immunoblotting, the indicated amounts of cell suspensions and purified SoxR protein standards were electrophoresed in SDS-polyacrylamide gels and transferred to nitrocellulose membranes with a TE series Transphor electrophoresis unit (Hoefer Scientific). The filters were probed with the affinity-purified antisera, and bound antibody was detected with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G antibodies (Promega).

EPR Spectroscopy

X-band EPR spectra of either purified SoxR^c proteins or cell suspensions were recorded at 20°K on a Bruker model ESP300 spectrometer maintained at constant temperature with an Oxford Instruments model ESR910 continuous-flow cryostat as described previously (Hidalgo and Demple, 1996a). The amount of reduced SoxR was determined by comparison in the same experiment to standardized Fe-SoxR samples after reduction with dithionite (Hidalgo et al., 1995, Hidalgo and Demple, 1996a). The high EPR background noise of the cell paste was greatly reduced by electronically subtracting the spectrum of TN5311-*pKEN2* cells, which do not express SoxR, from the spectra for SoxR-containing samples.

soxS Induction Experiments: β-Galactosidase Assays

Strains EH46, EH56, EH76, EH66, and EH86 transformed with plasmids *pSE380*, *pSXR*, *pTN101*, *pTN102*, or *pTN104* were inoculated into Luria broth (Miller, 1992) with 30 μg/ml of kanamycin, 30 μg/ml of streptomycin, and 100 μg/ml of ampicillin and incubated at 37°C for ~16 h with vigorous shaking. The overnight cultures were diluted 100-fold into 1 ml of fresh medium in duplicate tubes and incubated at 37°C for exactly 90 min. PQ was then added at a final concentration of 0.25 mM to one of each pair of tubes, and incubation continued for 60 min. The samples were then placed on ice. β-Galactosidase activity in SDS-$CHCl_3$–treated cells was determined as described by Miller (1992).

E. coli strains carrying a chromosomal *soxS′::lacZ* fusion (TN5311 or TN5315) and containing plasmids expressing either the wild-type (*pSXR*; Amábile-Cuevas and Demple, 1991) or a constitutive *soxR* allele (*pTN101*, *pTN102*, or *pTN104*; Nunoshiba and Demple, 1994) were used to study the ability of the constitutive protein to stimulate *soxS* transcription in vivo under aerobic or anaerobic conditions. The inoculum for both aerobic and anaerobic cultures was prepared by subculturing up to three consecutive times in 4.5 ml, capped tubes, filled almost completely with the cultures, and incubation at 37°C without shaking. For aerobic growth, the inoculum was then diluted 1:30 in fresh Luria broth containing 100 μg/ml ampicillin, and incubation continued for 2 hr on a shaker. One-milliliter aliquots of each culture were then placed into two tubes, and diamide was added to one of them to a final concentration of 0.5 mM. Incubation with shaking at 37°C then continued for 60 min. For anaerobic growth, the inoculum was diluted 1:15 into the capped tubes filled with Luria broth medium previously degassed with argon. Incubation proceeded at 37°C without shaking for 2 hr. Then diamide (0.5 mM) was added to the indicated cultures, and incubation continued for 60 min. β-Galactosidase activities of either aerobic or anaerobic cultures were assayed as described above (Miller, 1992).

The same β-galactosidase assay was used to determine the activities of cultures of strain TN5311 transformed with either *pKOXR* or *pTN101K*, *pTN102K*, or *pTN104K*, prior to the production of cell suspensions and EPR analysis (see above).

Redox Titration of Wild-Type and SoxR^c Proteins

To estimate the midpoint redox potential of the wild-type and SoxR^c proteins, samples of 2.5 ml of ~15 μM SoxR protein were equilibrated with argon for ~60 min at room temperature inside an anaerobic redox cuvette (Dutton, 1978; Ding et al., 1996) in the presence of the redox mediator safranine O, present at 3 μM ($E_0 = -289$ mV [pH 7.6]). The redox potential was monitored with a redox microelectrode permanently attached to the system (Microelectrodes Inc., Bedford, New Hampshire), and freshly prepared sodium dithionite solution or potassium ferricyanide was slowly added with a gas-tight Hamilton syringe, with a permanent argon flow and continuous stirring. The redox state of the [2Fe-2S] clusters of SoxR was followed at 414 nm, one of the maxima for oxidized SoxR (Hidalgo and Demple, 1994; Ding and Demple, 1996) with which the mediator safranine O does not interfere. Absorbance was recorded in a ultraviolet-visible spectrophotometer (Perkin-Elmer Lambda 3A).

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