The Redox State of the [2Fe-2S] Clusters in SoxR Protein Regulates Its Activity as a Transcription Factor

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SoxR protein is a redox-responsive transcription factor that governs a regulon of oxidative stress and antibacterial resistance genes in *Escherichia coli*. Purified SoxR contains oxidized [2Fe-2S] clusters and stimulates *in vitro* transcription of its target gene *soxS* up to 100-fold. SoxR transcriptional activity, but not DNA binding, is completely dependent on the [2Fe-2S] clusters; apo-SoxR prepared *in vitro* binds the *soxS* promoter with unchanged affinity but does not have transcription activity. Thus, modulation of the SoxR [2Fe-2S] clusters was proposed to control the protein's function in transcription. Here, we provide evidence that SoxR with reduced [2Fe-2S] clusters is inactive. Redox titration of purified SoxR revealed a midpoint potential of ~285 ± 10 mV (pH 7.6). *In vitro* transcription assays showed that SoxR was inactivated when the [2Fe-2S] cluster was reduced (~380 mV), and full activity was restored upon reoxidation (+100 mV). The results suggest that one-electron oxidation and reduction of the [2Fe-2S] cluster regulate SoxR transcriptional activity.

The *soxRS* regulon is a multifunctional cellular defense system against oxidative stress in *Escherichia coli* (1, 2). The regulon is controlled by two transcription factors, SoxR and SoxS, which act sequentially. SoxR is a constitutive protein that is activated post-translationally by superoxide stress (3, 4) or nitric oxide (5) to stimulate expression of the *soxS* gene. The increased amount of SoxS protein then stimulates expression of the regulon genes (6).

SoxR protein is a homodimer containing a pair of [2Fe-2S] clusters (7, 8) anchored to four cysteine residues in the C-terminal region of the polypeptide. Mutations changing any of these cysteines to alanine eliminates the [2Fe-2S] clusters from SoxR and prevents *in vivo* activation of the protein by the superoxide-generating agent paraquat. In *vitro* experiments with wild-type SoxR protein showed that disruption of the [2Fe-2S] clusters by thiols to generate apo-SoxR does not affect *soxS*-specific DNA binding but does eliminate transcriptional activity (9, 10). These results demonstrated that the [2Fe-2S] clusters are essential for the SoxR regulatory function both *in vivo* and *in vitro*.

The mechanism for SoxR activation is not established. One possibility is that assembly and disassembly of the SoxR [2Fe-2S] clusters could contribute to the regulation of SoxR transcriptional activity. It has been shown that glutathione-based free radicals disrupt the [2Fe-2S] clusters, thereby inactivating SoxR (10). Moreover, assembly of SoxR [2Fe-2S] clusters from Fe²⁺ and S²⁻ can occur rapidly *in vitro* (11), which is a prerequisite for such a possible regulatory role. Although assembly and disassembly of the [2Fe-2S] clusters must occur during SoxR metabolism, whether these processes are rate-limiting in the activation of SoxR *in vivo* has yet to be demonstrated.

An alternative regulatory mechanism proposed for SoxR is through reduction and oxidation of the [2Fe-2S] clusters (9). Although such an experiment was obvious to do, reoxidation of reduced SoxR [2Fe-2S] clusters during *in vitro* transcription reactions yielded inconsistent results. In this report, we have used a specially designed anaerobic redox cuvette to prevent reoxidation of reduced SoxR [2Fe-2S] clusters during the *in vitro* transcription assay. The results show that SoxR protein is reversibly inactivated when the [2Fe-2S] clusters are reduced.

MATERIALS AND METHODS

**Purification of SoxR Protein—**Purification of SoxR from *E. coli* containing the expression plasmid pKOXR was done as described previously (9, 10). The purity of SoxR protein used in this work was >90% as judged by staining of sodium dodecyl sulfate-polyacrylamide gels.

**Redox Titration of SoxR [2Fe-2S] Clusters—**A specially designed anaerobic redox cuvette was used for redox titrations as described by Dutton (12). Before titration, solutions containing SoxR protein were equilibrated with ultra-pure argon in anaerobic cuvette for about 1 h at room temperature. During titration, the argon flow was maintained with gentle stirring by a small magnet on the bottom of the cuvette. The redox potential of the solution was adjusted by adding freshly prepared sodium dithionite or potassium ferricyanide using a gas-tight 10-μl Hamilton microsyringe (Hamilton Co., Reno, NV). The redox potential was monitored directly with a redox microelectrode (Microelectrodes Inc., Bedford, NH).

The redox state of SoxR [2Fe-2S] clusters was monitored by absorbance at 414 nm (one of the maxima for oxidized SoxR; see Refs. 8 and 10) to minimize the absorbance interference from the redox mediator safranine O (present at 3 μM). After examining the absorbance spectra of reduced and oxidized SoxR [2Fe-2S] clusters, an approximate isosbestic point at 391 nm was chosen as the reference wavelength for the redox titration experiment. Absorbance was recorded in a UV-visible spectrophotometer (Perkin-Elmer Lambda 3A).

**Transcriptional Activity of SoxR Protein *in Vitro*—**SoxR activity was assayed by measuring *in vitro* transcription of the *soxS* gene in plasmid pBD100, which also contains the SoxR-independent control gene *bla* (5). Reaction transcription mixtures contained 20 nM SoxR protein, 10 nM pBD100 DNA, 1.25 mM each of ATP, GTP, CTP, and UTP, 75 mM KCl, 1 mM diithiothreitol, 10% glycerol, 15 mM MgCl₂, 100 mM Tris-HCl (pH 7.5), and 10 μM of redox mediator safranine O. The reaction mixtures were equilibrated with ultra-pure argon in anaerobic cuvette for about 1 h, and the redox potential of solution was adjusted and monitored as described above. After the redox potential was adjusted by the addition of dithionite for reduction or potassium ferricyanide for oxidation, al-

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1 T. M. Bradley, E. Hidalgo, H. Ding, and B. Demple, manuscript submitted.

2 H. Ding, E. Hidalgo, and B. Demple, unpublished observations.
Redox State of [2Fe-2S] Cluster Regulates SoxR Activity

Redox titration of the SoxR [2Fe-2S] clusters. SoxR protein (10 μM) in 50 mM N-2-hydroxyethylpiperazine-N’-2-ethane-sulfonic acid/NaOH buffer (pH 7.6), 500 mM NaCl was supplemented with 3 μM safranine O as a redox mediator (12). Redox titration was performed in an anaerobic redox cuvette as described under "Materials and Methods." The x axis shows the redox potential measured with microelectrode. The y axis shows the absorbance difference between 414 and 391 nm, normalized to 0 or 100% for fully reduced or oxidized SoxR, respectively. The redox potential was measured against a standard hydrogen electrode. The solid line drawn through the data points was generated from the Nernst equation with $n = 1$. The experiment was repeated three times. The mean value for redox midpoint potential was $-285 \pm 10$ mV.

Redox titration of SoxR [2Fe-2S] clusters—For [2Fe-2S] clusters, two redox states ($Fe^{3+}-Fe^{3+}$ and $Fe^{3+}-Fe^{3+}$) are generally considered under physiological conditions. The reduced form ($Fe^{0}-Fe^{0}$) is paramagnetic and has a distinct EPR spectrum in SoxR (7, 8, 10). The oxidized form ($Fe^{3+}-Fe^{3+}$) is diamagnetic but has an optical absorbance spectrum typical for [2Fe-2S] clusters (7, 8, 10). These characteristics allowed us to monitor the redox state of SoxR [2Fe-2S] clusters directly. For the redox titration of SoxR protein, we monitored the optical absorbance spectrum of SoxR.

A typical redox titration of the SoxR [2Fe-2S] cluster at pH 7.6 is shown in Fig. 1. Each redox potential was equilibrated for $\geq 2$ min before absorbance measurement. The solid line drawn through the data points is the theoretical calculation of the Nernst equation with $n = 1$. The midpoint potential for the SoxR [2Fe-2S] clusters under these conditions was estimated to be $-285 \pm 10$ mV. It is worth noting that this value is close to the intracellular redox potential estimated for E. coli during exponential growth (13).

SoxR with Reduced [2Fe-2S] Clusters Is Inactive in Vitro—Redox titration of the in vitro transcription activity of SoxR protein was done under the conditions described above, except that SoxR protein was present at a 500-fold lower concentration. Fig. 1 shows the result of in vitro transcription under various redox potentials. The synthesis of the SoxR-dependent soxS transcript was dramatically decreased ($-20$-fold) when the redox potential was adjusted from +200 to $-380$ mV (where the SoxR [2Fe-2S] clusters are $>95\%$ reduced; see Fig. 1). When the redox potential was increased from $-380$ mV back to $+100$ mV (where the SoxR [2Fe-2S] clusters are reoxidized; Fig. 1), SoxR-dependent soxS transcription was fully restored (Fig. 2). In contrast, the amount of the SoxR-independent bla transcript remained virtually unchanged as the redox potential was varied from +200 to $-380$ mV and back to $+100$ mV. Thus, the α70 RNA polymerase was not significantly affected over the range of redox potentials used in these experiments. These results clearly show that reduced SoxR is specifically inactive for soxS transcription and that SoxR activity is restored by reoxidation of the [2Fe-2S] clusters.

**DISCUSSION**

Activated SoxR bound to DNA exerts structural changes in the soxS promoter that compensate for the $-70^\circ$ overwinding of the $-10$ and $-35$ promoter elements (8, 9). A key question is how this structural effect is transmitted via changes in the oxidation state of SoxR [2Fe-2S] clusters. Like many other DNA binding proteins, SoxR is a homodimer and binds a symmetric DNA site. Gel filtration chromatography under denaturing conditions indicates that each SoxR monomer contains a single [2Fe-2S] cluster and that apo-SoxR is also homodimeric (9). Thus, the association between two SoxR monomers is not mediated by the [2Fe-2S] clusters, and oxidation would involve the loss of one electron from each SoxR monomer. One mechanism driving a significant conformational change in the protein would be electrostatic repulsion between the two positive charges, especially if the two [2Fe-2S] clusters are physically close. Because Fe is not required for DNA binding by SoxR (9) and the reduced [2Fe-2S] clusters of SoxR are stable (7, 8), SoxR activation seems to be an electron-driven conformational change in the DNA-protein complex.

The E. coli intracellular redox potential is estimated to be $-260$ to $-280$ mV (15), a value close to the redox midpoint potential of the SoxR [2Fe-2S] clusters determined in vitro ($-285 \pm 10$ mV; Fig. 1). If these values were directly comparable, about half of the SoxR in cells would be in the oxidized state. However, the in vivo estimates of redox potential are indirect, and intracellular conditions (e.g. pH) could also shift the midpoint potential of SoxR [2Fe-2S] clusters to a higher value than found in vitro. In the range we are considering, a cellular redox potential 60 mV lower than that of the SoxR [2Fe-2S] clusters would maintain $>90\%$ of the SoxR protein in the reduced (inactive) state in vivo.

Oxidative activation of SoxR in vivo could occur in various ways. In cells challenged with redox cycling agents such as paraquat, NADPH and perhaps other reductants could be ex-
hausted, increasing the intracellular redox potential and indirectly the proportion of oxidized [2Fe-2S] clusters in SoxR. Consistent with this hypothesis, Fridovich and Liochev noted that SoxR is more readily activated by paraquat in bacteria deficient in glucose-6-phosphate dehydrogenase, an enzyme expected to contribute to the NADPH pool (14). This mechanism does not posit a direct role for superoxide as a signal, but deficiency in superoxide dismutase activates SoxR in the absence of paraquat (4). Moreover, SoxR is activated by nitric oxide more dramatically under anaerobic than aerobic conditions (5). Thus, superoxide and nitric oxide could also be direct oxidants of the SoxR [2Fe-2S] clusters to activate the system. It also seems likely that the [2Fe-2S] clusters of SoxR are maintained in the reduced state by an enzymatic reductase yet to be identified. Thus, a third possibility is that the putative reductase is inactivated by superoxide or nitric oxide to allow oxidized SoxR to accumulate. As this manuscript was being completed, we learned of independent studies that indicate reversible inactivation of SoxR in vitro by reduction of its [2Fe-2S] clusters (15).

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