Binuclear [2Fe-2S] Clusters in the Escherichia coli SoxR Protein and Role of the Metal Centers in Transcription*

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SoxR protein of Escherichia coli is activated by superoxide-generating agents or nitric oxide as a powerful transcription activator of the soxS gene, whose product activates ~10 other promoters. SoxR contains non-heme iron essential for abortive initiation of transcription in vitro. Here we show that this metal dependence extends to full-length transcription in vitro. In the presence of E. coli σ70 RNA polymerase, iron-containing SoxR mediates open complex formation at the soxS promoter, as determined using footprinting with Cu-5-phenyl-1,10-phenanthroline. We investigated the nature of the SoxR iron center by chemical analyses and electron paramagnetic resonance spectroscopy. Dithionite-reduced Fe-SoxR exhibited an almost axial paramagnetic signature with g values of 2.01 and 1.93 observable up to 100 K. These features, together with quantitation of spin, iron, and S²⁻ and hydrodynamic evidence that SoxR is a homodimer in solution, indicate that (SoxR)₂ contains two [2Fe-2S] clusters. Treatment of Fe-SoxR with high concentrations of dithiothreitol caused subtle changes in the visible absorption spectrum and blocked transcriptional activity without generating reduced [2Fe-2S] centers, but was also associated with the loss of iron from the protein. However, lowering the thiol concentration by dilution allowed spontaneous regeneration of active Fe-SoxR.

Excessive production or inadequate disposal of reactive derivatives of oxygen, such as superoxide (O₂⁻) and hydrogen peroxide (H₂O₂), is called oxidative stress (Sies, 1991). Cells respond to sublethal levels of oxidative stress by coordinately activating batteries of antioxidant genes (Demple, 1991; Hidalgo and Demple, 1995). The molecular signals that activate these multifunctional defense systems have been the objects of considerable recent interest (Meyer et al., 1993; Gounalaki and Thireos, 1994; Nunoshiba et al., 1994; Storz et al., 1995). In contrast, the soxRS system governs an inducible response to superoxide-generating agents (Nunoshiba et al., 1992; Wu and Weiss, 1992) or nitric oxide (Nunoshiba et al., 1993, 1995). The soxRS response occurs in two stages: an intracellular signal of oxidative stress converts existing SoxR protein into a potent transcriptional activator of the soxS gene; the resulting increase in SoxS levels then triggers expression of the various regulon genes (Amábile-Cuevas and Demple, 1991).

SoxR, isolated from bacteria that overproduce the protein, contains an FeS cluster(s) essential for in vitro transcriptional activation of the soxS promoter (Hidalgo and Demple, 1994). The metal is not required for the binding of SoxR to the soxS promoter nor for the subsequent binding of RNA polymerase (Hidalgo and Demple, 1994). These observations suggest that the critical effect of activated SoxR on transcription occurs at a later stage, perhaps by specific conformational effects of Fe-SoxR²⁺ on DNA, as proposed for the homologous MerR protein (Ansari et al., 1992). It seems likely that the FeS center(s) of SoxR is involved in the signal transduction mechanism that links O₂⁻ or NO⁺ stress to gene activation in the soxRS system.

Although protein FeS centers have been most commonly associated with electron transfer and some enzymatic dehydration reactions (Johnson, 1994), iron has recently been proposed as an important regulatory component of other genetic responses (Beinert, 1990). In the cytoplasmic form of mammalian aconitase, for example, the iron center regulates the activity of the protein as an RNA-binding factor: the apoprotein binds mRNAs encoding ferritin (blocking its translation) and the transferrin receptor (stabilizing the message), while the [4Fe-4S] and [3Fe-4S] forms do not (Klausner et al., 1993). This protein is thus linked to the iron status of the cell and coordinates key constituents of iron assimilation, utilization, and storage. The Fnr protein of E. coli coordinates transcription of a large number of genes that allow cells to take advantage of electron acceptors other than oxygen (Green and Guest, 1993). Although iron seems to be required for in vitro DNA binding by Fnr, the structure of its metal center has not been elucidated.

The structure of the iron center in SoxR is of importance both for the transcriptional activity described above and for the role of this protein as a sensor of oxidative stress. We describe here experiments that define [2Fe-2S] clusters in SoxR, and we show that only the metallolprotein activates in vitro transcription and is apparently associated with formation of the open complex at the soxS promoter.

*The abbreviations used are: Fe-SoxR, iron-containing SoxR; CuPPA, 5-phenyl-1,10-phenanthroline-copper(I); DTT, dithiothreitol; FeS, iron-sulfur; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s).

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MATERIALS AND METHODS

Purification of SoxR—Purification of SoxR protein from E. coli containing the SoxR expression plasmid pXOR was performed as described previously (Hidalgo and Demple, 1994). Fractions eluted from heparin-agarose columns (Life Technologies, Inc.) (purity of ~65–80%) were used for these studies; the more purified material obtained by DNA-affinity chromatography (Hidalgo and Demple, 1994) could not be obtained in a high enough concentration for the physical studies conducted.

In Vitro Transcription—All the solutions and reagents used during the assay were prepared RNase-free (Sambrook et al., 1989). Protein-DNA binding reactions were performed in 19 μl containing 75 mM KCl, 2 mM diithiothreitol (DTT), 10 mM glyceral, 15 mM MgCl2, 10 mM Tris-HCl, pH 7.5, ~200–400 ng of pBD100 DNA, and the indicated amounts of SoxR. Plasmid pBD100 is a pBR322 derivative containing an E. coli genomic insert of 4 kb that includes the whole soxS gene (Amaña-Cuevas and Demple, 1991). After a 5-min incubation at room temperature, 1 μl of 1 mg/ml E. coli RNA polymerase containing DNA-reactive polymerase RNA polymerase (~300 units/ml; kindly provided by Drs. Linda D’Ari and Michael Chamberlin, University of California, Berkeley) was added to each sample to achieve a final concentration of 0.1 μM, and the reactions were incubated for 15 min at 37°C. One μl of a mixture of the four NTPs (25 μM each) was then added, and the polymerase extension reaction proceeded for another 5 min at 37°C. The in vitro transcription reaction was then stopped by mixing into each sample 330 μl of a mixture of a solution of 73% ethanol, 7 μg/ml RNA, 0.11 mM sodium acetate. After a 45-min precipitation at −20°C, the samples were centrifuged for 20 min at 10,000 × g at room temperature. The precipitates were resuspended in 10 μl of 0.1 M NaCl, 50 mM HEPES, pH 7.6. The relatively high salt concentration was necessary to maintain SoxR solubility. Bovine carbonic anhydrase (29.0 kDa; Sigma) and soybean trypsin inhibitor (20.1 kDa; Sigma) were used as markers. After sedimentation for 24 h at 50,000 rpm in a Beckman SW50.1 rotor, 0.3-ml fractions were collected from the bottoms of the tubes using a peristaltic pump. Samples were analyzed by SDS-PAGE, silver staining, and quantitative densitometry (BioImage system, Millipore).

Protein, Iron, and Labile Sulfide Determinations—Protein concentrations were determined by Coomassie staining of SDS-polyacrylamide gels using as a standard SoxR previously quantified by amino acid analysis (Hidalgo and Demple, 1994).

Iron concentration was determined by two different methods: by inductively coupled plasma emission spectrometry (Hidalgo and Demple, 1994) and colorimetrically by using the iron chelator ferrozine (Stokey, 1970). For the colorimetric determinations, 460-μl samples of SoxR were mixed with 100 μl of ultrapurified concentrated HCl (Baker analyzed), and the mixture was incubated at 80°C for 20 min with occasional vigorous shaking. After centrifugation at 15,000 × g for 5 min to remove denatured protein, 510 μl of the supernatant was mixed with 20 μl of 10 mM ferrozine and 20 μl of 75 mM ascorbic acid. The mixture was neutralized by the addition of 120 μl of saturated ammonium acetate to allow ferrozine chelation. After a 20-min incubation at room temperature, the absorbance at 562 nm was determined and iron concentration was calculated using ε_ferrozine=Fe = 27,900 M−1 cm−1 (Stokey, 1970).

Labile inorganic sulfide was determined by using a modification of the basic methylene blue procedure (Fogo and Popowsky, 1949) as described by Beinert (1983).

Electron Paramagnetic Resonance (EPR) Spectroscopy—Samples of purified Fe-SoxR (500 μl at ~10 μM) inside an anaerobic chamber (Vacuum Atmospheres Co. model HE-493) at an O2 concentration of ~0.5–1 ppm were incubated with 10 μl of 3 mM dithionite (freshly dissolved; this amount contained ~25 reducing equivalents (Hidalgo and Demple, 1994)). Oxidized and reduced SoxR were sealed in cuvettes and removed from the chamber, and the spectra were recorded with a Hewlett-Packard model 8452A spectrophotometer to determine the extent of reduction. The cuvettes were then returned to the anaerobic chamber, vacuum-dried, and 300 μl of pyridine was added to each EPR sample tube. Tubes were sealed with rubber septa, removed from the anaerobic chamber, and immediately frozen in a bath of liquid nitrogen until analysis. EPR spectra at X-band were recorded at 10–100 K on a Bruker model ESP 300 spectrometer maintained at constant temperature either with an Oxford Instruments ESR 910 continuous flow cryostat or with a Bruker ER411VT variable temperature controller. Spin quantitations were carried out under nonsaturating conditions and were calculated by comparison of the integrated intensity of samples with that of a 1 mM Cu2+ prepared as described by Malmström et al. (1970). Intensities of samples and the standard were corrected for differences in g value as described previously (Aasa and Vännärd, 1976).

DTT Treatment of Purified Fe-SoxR Fractions—SoxR protein was incubated aerobically with increasing concentrations of DTT (10 to 100 mM) for 10 min at room temperature. The spectra of the oxidized and DTT-treated preparations were recorded in a Perkin-Elmer Lambda 3A UV/VIS spectrophotometer. In vitro transcription reactions for the DTT-treated samples were performed under the conditions described above, except that the same concentration of DTT used for the SoxR protein treatment was maintained in the transcription reaction. Reversibility of the DTT treatment was analyzed by omitting DTT from the in vitro transcription reaction. Stability of the Fe5 center in DTT-treated SoxR was analyzed by gel filtration chromatography of 100-μl samples using NICK columns (Pharmacia), following the manufacturer’s instructions. Protein concentration in each 15-μl sample was measured as described above. Colorimetric determination of iron was performed by adding 0.1 μl DTT to the eluted fractions (except for those which already contained it), and a further addition of 10 μl of 10 mM ferricron. The absorption at 562 nm was determined after 20 min of incubation, and iron concentrations were estimated as described above.

RESULTS

Fe-SoxS-Dependent Transcription on a Supercoiled Template—We previously showed that purified Fe-SoxR, but not apo-SoxR, was able to initiate abortive transcription from a linear template containing the soxS promoter (Hidalgo and Demple, 1994). However, we had not examined whether this difference is retained in the more relevant situation of a supercoiled template and transcription of the intact soxS gene in the
presence of competing promoters. A supercoiled plasmid containing the whole soxRS locus (pBD100; Amábile-Cuevas and Demple, 1991) was used as the template for these in vitro reactions and allowed for the simultaneous detection of bla (β-lactamase gene) transcription, which was expected to be SoxR-independent. In the presence of E. coli σ70-containing RNA polymerase and Fe-SoxR, a 64-nucleotide primer extension product was detected that corresponded to the size expected for soxS mRNA, while a 105-nucleotide product corresponded to the size expected for the bla transcript. In these experiments (Fig. 1), the amount of soxS mRNA increased strongly with the amount of Fe-SoxR added to the in vitro transcription reaction and was near-maximal with 10 ng of this protein. In contrast, even with 100 ng of apo-SoxR, only a small increase in the production of soxS transcript was observed compared to control reactions with RNA polymerase alone. Thus, the transcriptional activity of SoxR in this assay also depends strongly on its iron content.

5-Phenyl-1,10-phenanthroline-Copper(I) Footprinting—Our previous studies (Hidalgo and Demple, 1994) showed that Fe-SoxRhasonlyasmallsignificant effect on the binding of RNA polymerase to the promoter, which suggested that Fe-SoxR acts at a subsequent step, such as conformational changes leading to the initiation of transcription. We addressed this issue by conducting footprinting studies with 5-phenyl-1,10-phenanthroline-copper(I) (CuPPA), a reagent that is also sensitive to protein-induced changes in DNA structure (Sigman et al., 1991). Footprints of both Fe-SoxR and apo-SoxR across the 210 to 235 region of both strands of the soxS promoter were apparent in the absence and in the presence of RNA polymerase (Fig. 2A). These footprints were not as sharply defined as those

![Footprint Image](image-url)
observed with DNase I (Hidalgo and Demple, 1994), but were detected consistently.

CuPPA-hypersensitive sites were observed in the vicinity of the soxS transcription start site only with Fe-SoxR and RNA polymerase together, in both the nontranscribed (Fig. 2A, left) and the transcribed strand (Fig. 2A, right). CuPPA-hypersensitive sites at -3 to -7 in the transcribed strand and at +4 and +5 of the nontranscribed strand have been associated with open complex formation at other promoters (Sigman et al., 1991; Thederahn et al., 1990). CuPPA-hypersensitive sites were also reported at +4 to +6 in the transcribed strand of the lacUV5 open complex (Thederahn et al., 1990), but were not apparent in our experiments. These sites may have been difficult to detect because they are much weaker than those seen at -3 to -7 in the same strand (Sigman et al., 1991; Thederahn et al., 1990). Taken together, these data indicate that Fe-SoxR specifically leads to open complex formation by RNA polymerase.

In addition, three sites in the nontranscribed strand, in the center of the SoxR binding site, became unprotected against CuPPA only with Fe-SoxR and RNA polymerase together (Fig. 2A, left side). This deprotection was observed consistently when the background cleavage by CuPPA was high, but was difficult to detect under milder cleavage conditions. We therefore determined whether the footprinting reagents had specific effects on the in vitro transcription of soxS. CuPPA at 0.16 mM inhibited soxS-specific transcription (relative to bla transcription) by 30%, but 6.7 mM mercaptopropionic acid eliminated detectable transcription of both bla and soxS. Because of the general inhibition of transcription by mercaptopropionic acid, we checked for specific effects on Fe-SoxR. Fe-SoxR retained 90% of the visible absorption characteristic of the intact metallocprotein (Hidalgo and Demple, 1994). Therefore, the integrity of Fe-SoxR was not strongly compromised by the footprinting reagents, although partial effects were noted.

The CuPPA footprinting results are compared in Fig. 2B with those found previously for DNase I (Hidalgo and Demple, 1994). The cleavage sites in the nontranscribed strand that became unprotected with Fe-SoxR and RNA polymerase are in the center of dyad symmetry of the SoxR binding site (Fig. 2B). Interestingly, CuPPA-hypersensitive sites were described (Hidalgo and Demple, 1994) and analyzed for SoxR protein Fe, S

Iron and S

2-

Content of Dimeric SoxR—Before determining the iron stoichiometry of SoxR, we first established the oligomeric state of the protein. Two features seemed to indicate that SoxR could exist in solution as a dimer (or higher oligomer). First, the SoxR homologue, MerR, is dimeric both in the presence and in the absence of the Hg

2-

(Helman et al., 1990). Second, SoxR binds a DNA site in the soxS promoter that has perfect dyad symmetry (Hidalgo and Demple, 1994). This hypothesis was tested by sedimentation analysis of both Fe- and apo-SoxR. In these experiments, a clear peak of SoxR protein was observed sedimenting just slower than carbonic anhydrase (29 kDa) and significantly faster than soybean trypsin inhibitor (20.1 kDa). After centrifugation, fractions were collected and analyzed for protein by SDS-PAGE, silver staining, and quantitative densitometry.

Table I: Iron and S

2-

content of SoxR

| SoxR | Fe | S

2- | Spin |
|------|----|------|
| µM  | µM | µM   | µM   |
| 10.6 | 27.9 | 14.0 | 12.7 | 2:6:1.3 |
| 21.1 | 62.8 | 34.0 | 26.6 | 1:3:0.16 |
| 8.6  | 28.8 | 16.8 | ND  | 1:3:2.0 |
| 24.9 | 63.0 | 44.1 | 28.6 | 2:5:1.8 |
| 16.0 | 41.7 | 29.5 | ND  | 2:6:1.8 |

Table I and data not shown). Together with the iron determinations described above, these data indicate that (SoxR)

6 might contain either a single [4Fe-4S] cluster or a pair of [2Fe-2S] centers.

EPR Spectroscopy of Fe-SoxR—EPR spectroscopy was used
The studies presented here indicate that SoxR protein is a homodimer that, in its activated form, contains a pair of [2Fe-2S] centers. These metal clusters are not required to maintain the overall structure of SoxR, since the apoprotein is also a homodimer that binds the SoxS promoter with high affinity (Hidalgo and Demple, 1994). It remains to be established whether the two SoxR [2Fe-2S] clusters are arranged as one per subunit or as a pair of clusters coordinated between the subunits. With respect to the latter possibility, it is interesting to note that a single Hg is coordinated to the homologous MerR protein by distinct cysteine ligands from each subunit of a dimer (Helmann et al., 1990). Two of these cysteine residues of MerR are positioned identically in alignments with the SoxR protein (Amálie-Cuevas and Demple, 1991). At least one intersubunit iron-sulfur cluster has been described, the [4Fe-4S] center of Azotobacter vinelandii nitrogenase (Georgiadis et al., 1992).

The [2Fe-2S] centers of SoxR are necessary for the protein's transactivation. Studies using gel filtration chromatography in the absence or presence of 100 mM DTT in the column buffer showed that the DTT concentration was diminished by gel filtration chromatography in elution buffer lacking the thiol (data not shown) or by omitting the DTT from the transfection reaction (Fig. 5B). Fe-SoxR transcriptional activity was completely recovered. The high DTT concentrations did not affect the transcription of the bla gene (Fig. 5B).

Although the visible absorption spectrum of Fe-SoxR was changed by the DTT treatment (Fig. 5A), DTT-treated SoxR did not show an EPR spectrum (data not shown). Therefore, DTT-treated Fe-SoxR does not correspond to the form of the protein with reduced [2Fe-2S] centers. We further analyzed the effect of DTT on transcription activity of Fe-SoxR by gel filtration chromatography in the presence or absence of 100 mM DTT in the column buffer. The amounts of SoxR protein and Fe were determined in the eluted fractions. SoxR and Fe co-eluted at a 1:2 ratio in the samples without DTT (Fig. 5C). Chromatography in the presence of DTT yielded a peak of SoxR associated with diminished amounts of iron (a 1:0.9 ratio in the experiment shown) accompanied by a considerable amount of iron of slower mobility (Fig. 5C). These data indicate that the effect of DTT reversibly blocks the transcriptional activity of Fe-SoxR by destabilizing the [2Fe-2S] centers, which can then be physically removed in the continuing presence of high levels of DTT.

**DISCUSSION**

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function as a transcriptional activator. The features of CuPPA footprinting of Fe-SoxR and RNA polymerase at the soxS promoter show that only active SoxR leads to open complex formation by the polymerase. For the homologous MerR protein, transcriptional activation has been proposed to result from localized underwinding that compensates for the suboptimal spacing (19 bp) between the 210 and 235 elements of the merT promoter (Ansari et al., 1992). The SoxR-regulated soxS promoter also seems to be overwound, with 19-bp spacing (Hidalgo and Demple, 1994).

The FeS centers of many proteins are damaged when cells are exposed to intracellular superoxide-generating agents or to nitric oxide. Superoxide-sensitive FeS proteins typically contain [4Fe-4S] centers (Gardner and Fridovich, 1991; Liochev and Fridovich, 1992). Tetranuclear FeS centers, as found in aconitase, are inactivated in mammalian cells exposed to nitric oxide (Drapier et al., 1993; Weiss et al., 1993), perhaps by peroxynitrite (Hausladen and Fridovich, 1994) formed from the combination of NO and O2 (Koppenol et al., 1992). In contrast, Fe-SoxR must remain active when E. coli is exposed to high intracellular fluxes of O2 (Nunoshiba et al., 1992; Wu and Weiss, 1992) or NO (Nunoshiba et al., 1993, 1995). Perhaps binuclear [2Fe-2S] clusters are well suited to this requirement. The [2Fe-2S] clusters of SoxR seem to be quite stable in the oxidized form that is rapidly generated upon exposure of the protein to air (Hidalgo and Demple, 1994). Such stability is shared by the spinach dihydroxyacid dehydratase, which has a [2Fe-2S] cluster that is stable in the presence of O2, while the [4Fe-4S] cluster of the E. coli dehydratase is exquisitely sensitive to O2 (Flint et al., 1993).

Although the metal centers of SoxR are clearly essential for the transcriptional activity of the protein (Hidalgo and Demple, 1994; this work), the mechanism that activates SoxR in vivo is unknown. As demonstrated by the EPR experiments present here, the oxidized form of the protein is certainly active. The question then is whether the nonactivated state for SoxR is the reduced form or the apoprotein, or perhaps some other species. Some recent experiments suggest that dithionite-reduced Fe-SoxR is still active as a transcription factor (in contrast to DTT-treated Fe-SoxR), but the ease with which this protein is reoxidized by O2 in vitro indicates that caution should be applied in this interpretation. It is also possible that apo-SoxR is the physiologically relevant inactive state. If this is so, the [2Fe-2S] centers would be reconstituted adventitiously during extraction of the protein, even from cells not treated to activate SoxR. This possibility is being explored.

So far, apo-SoxR has been isolated only following purification of the protein in buffers containing β-mercaptoethanol (Hidalgo and Demple, 1994). Since the mere addition of β-mercaptoethanol to Fe-SoxR did not affect its spectroscopic or transcriptional properties, it seemed likely that this thiol destabilizes the [2Fe-2S] centers and allows their removal during chromatography (Hidalgo and Demple, 1994). A similar destabilizing effect seems to be mediated by DTT, high concentrations of which also abolish the transcriptional activity of Fe-SoxR. DTT-treated Fe-SoxR and apo-SoxR are the only two transcriptionally inactive forms of the protein thus far identified. This opens the possibility that the DTT-treated protein could correspond to the inactive form of SoxR in vivo, perhaps with partially disassembled [2Fe-2S] centers. However, the high thiol

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2 E. Hidalgo and B. Demple, unpublished data.
concentrations necessary to generate this inactive state do not prevail in cells, which typically contain ~5 mM glutathione accounting for most of the low molecular weight thiol (Meister and Anderson, 1983).

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