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An iron–sulfur center essential for transcriptional activation by the redox-sensing SoxR protein

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The *soxRS* oxidative stress regulon of *Escherichia coli* is triggered by superoxide ($O_2^{\cdot-}$) generating agents or by nitric oxide through two consecutive steps of gene activation. SoxR protein has been proposed as the redox sensing gene activator that triggers this cascade of gene expression. We have now characterized two forms of SoxR: Fe-SoxR contained non-heme iron (up to 1.6 atoms per monomer); apo-SoxR was devoid of Fe or other metals. The spectroscopic properties of Fe-SoxR indicated that it contains a redox active iron–sulfur (FeS) cluster that is oxidized upon extraction from *E.coli*. Fe-SoxR and apo-SoxR bound the *in vivo* target, the *soxS* promoter, with equal affinities and protected the same region from DNase I *in vitro*. However, only Fe-SoxR stimulated transcription initiation at *soxS in vitro* >100-fold, similar to the activation of *soxS* expression *in vivo*. This stimulation occurred at a step after the binding of RNAP and indicates a conformational effect of oxidized Fe-SoxR on the *soxS* promoter. The variable redox state of the SoxR FeS cluster may thus be employed *in vivo* to modulate the transcriptional activity of this protein in response to specific types of oxidative stress.

Key words: adaptive response/DNA binding/*Escherichia coli*/oxidative stress/signal transduction

Introduction

Cells adapt to changing and sometimes stressful environments through specific genetic responses. The molecules that signal these extracellular and intracellular changes are known in only a few cases, primarily in prokaryotic systems. Examples are induction by allolactose of the *Escherichia coli lac* operon (Beckwith, 1987), cAMP to signal glucose deprivation (Kolb *et al.*, 1993), tryptophan to signal an abundance of this amino acid (Yanofsky, 1984) or Hg^{2+} to signal its own toxic presence (Summers, 1992). For these systems, the effector molecule is bound (usually reversibly) by the regulatory protein itself to inactivate (*lac* repressor) or activate (*trp* repressor) DNA binding, or to trigger a dormant transcription activating function (CRP or MerR proteins). Indirect control, as in the two component sensor/regulator protein pairs common in prokaryotes (Parkinson and Kofoid, 1992), may also predominate in eukaryotes (Karin and Smeal, 1992).

Genetic responses to oxidative stress occur in all kinds of organisms, but little is known about the sensing and regulatory systems involved. Oxidative stress arises when the physiological balance is upset between production and scavenging of unstable oxygen derivatives (Sies, 1991). These reactive species are, in order of sequential reduction from O_2, superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$), which can be generated by normal aerobic metabolism (Chance *et al.*, 1979) or by external sources such as near UV radiation (Keyse and Tyrrell, 1989) or agents such as paraquat (PQ), which divert electrons to molecular oxygen to generate $O_2^{\cdot-}$ and deplete the cellular pools of NAD(P)H in a process called redox cycling (Kappus and Sies, 1981). Oxidative stress has been closely related to degenerative diseases such as cancer (Cerutti, 1985), atherosclerosis (Frei *et al.*, 1989) or, recently, amylotrophic lateral sclerosis (Rosen *et al.*, 1993), and to the normal aging process (Ames, 1989; Harman, 1991). The ability of cells to respond to oxidative stress could be an important mitigating factor for these age-dependent processes.

In mammalian cells, responses to oxidative stress include the activation of a heme oxygenase (Keyse and Tyrrell, 1989) and an apparent tyrosine phosphatase gene (Keyse and Emslie, 1992) and probably of NF-κB dependent promoters (Meyer *et al.*, 1993). The yeast *Saccharomyces cerevisiae* exhibits two distinct protective responses to hydrogen peroxide and to $O_2^{\cdot-}$ generating agents (Jamieson, 1992; Flattery-O'Brien *et al.*, 1993). Such distinct, but overlapping, responses were previously known in bacteria (Demple and Halbrook, 1983; Christman *et al.*, 1985; Greenberg and Demple, 1989), where more information has become available about the actual sensors of oxidative stress (Demple, 1991; Farr and Kogoma, 1991).

In *E.coli*, the presence during aerobic growth of $O_2^{\cdot-}$ generating or redox cycling agents such as PQ induces the synthesis of ~40 proteins that are not inducible by H_2O_2 (Greenberg and Demple, 1989). At least nine of these proteins are controlled by *soxRS*, a two gene locus at 92.2 min on the *E.coli* chromosome (Greenberg *et al.*, 1990; Tsaneva and Weiss, 1990). Roles in defense against $O_2^{\cdot-}$ stress have been assigned for many of these proteins (Demple, 1991). Proteins induced under *soxRS* control include: Mn-containing superoxide dismutase (MnSOD), encoded by *sodA*, which scavenges intracellular $O_2^{\cdot-}$; glucose-6-phosphate dehydrogenase, encoded by *zwf*, which replenishes the NAD(P)H that redox cycling consumes; endonuclease IV, encoded by *nfo*, which repairs radical generated DNA damage; FumC, a redox resistant fumarase encoded by *fumC*, which replaces the oxidant sensitive form (Liochev and Fridovich, 1992).

The *soxRS* regulon is also activated by the free radical nitric oxide (Nunoshiba *et al.*, 1993). The sensing of this gas provides *soxRS* dependent bacterial resistance to the onslaught of nitric oxide generating macrophages. This resistance is due to at least some of the antioxidant functions listed above (Nunoshiba *et al.*, 1993).

The *soxRS* regulatory locus encodes two proteins, SoxR and SoxS (Amábile-Cuevas and Demple, 1991; Wu and Weiss, 1991), that effect a novel type of two step

transcriptional regulation. In this model (Demple and Amábile-Cuevas, 1991), a redox signal produced by $O_2^{\cdot -}$ generating agents activates pre-existing SoxR protein. The activated SoxR then triggers transcription of the *soxS* gene (Nunoshiba *et al.*, 1992; Wu and Weiss, 1992). The newly synthesized SoxS, a 13 kDa protein related to the AraC family of prokaryotic activators, is the proximal activator of the *soxRS* regulon genes (Amábile-Cuevas and Demple, 1991).

The 17 kDa SoxR protein therefore may be both a sensor for $O_2^{\cdot -}$ stress and a transcriptional activator of the *soxS* gene. SoxR protein is homologous to the MerR family of transcriptional activators (Amábile-Cuevas and Demple, 1991), which in the presence of Hg^{2+} trigger the expression of the *mer* operon, involved in the metabolism of this toxic metal (Summers, 1992). The homology spans an N-terminal helix−turn−helix motif involved in DNA recognition and binding in MerR (Ross *et al.*, 1989), and part of a cluster of cysteine residues by which MerR binds Hg^{2+}. Nevertheless, SoxR is unresponsive to heavy metals (Nunoshiba *et al.*, 1992).

The ability of SoxR to sense specific signals of oxidative stress indicated that fundamental information would accrue from a detailed study of this protein. We therefore sought molecular information on gene activation and redox signalling by isolating and analyzing the SoxR protein, which we show here to be a powerful transcription factor whose activity depends on a tightly bound iron−sulfur center.

Results

Purification of SoxR protein

In previous work (Amábile-Cuevas and Demple, 1991; Nunoshiba *et al.*, 1992) we developed expression systems for the overproduction of SoxR protein. After induction with IPTG, cells bearing a construct driven by a *lacI*-controlled *trp−lac* hybrid promoter express ~5% of the total cellular protein as soluble SoxR (Nunoshiba *et al.*, 1992) and provided the starting material for the isolation of SoxR. In some cases, the cells were treated with PQ (1 mM for 60 min) prior to extraction in order to activate SoxR *in vivo*. SoxR was detected by virtue of its specific DNA binding activity (Nunoshiba *et al.*, 1992) and as a 17 kDa polypeptide in SDS−polyacrylamide gels (Figure 1).

A simple procedure was developed to isolate SoxR protein (Table I; see Materials and methods for details). Soluble proteins were extracted from the bacteria using a French pressure cell, and the extracts clarified by centrifugation. After the removal of nucleic acids and some anionic proteins by passage through a DEAE column, SoxR was bound to a heparin−agarose column and eluted at relatively high ionic strength. This material (fraction III) ranged from 60 to 80% pure in SoxR and was suitable for many studies. Essentially homogeneous SoxR was obtained by a further processing of fraction III on affinity columns containing a DNA fragment with the *soxS* promoter, to yield fraction IV (purity >95%). This protocol was similarly effective whether or not the purification buffers contained the reducing agent β-mercaptoethanol (βME) (Table I). The DNA binding activity of SoxR was stable throughout the purification and the protein could be stored for ≥6 months at −20°C without apparent loss of this activity. The identity of SoxR protein in fraction IV was confirmed by sequencing the first three residues of the polypeptide N terminus (Met-Glu-Lys).

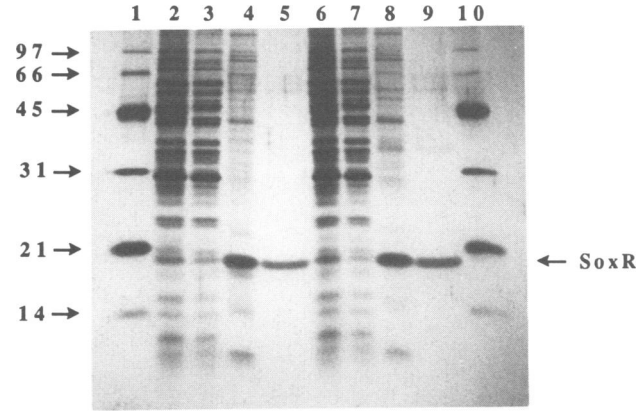

Fig. 1. Electrophoretic analysis of SoxR fractions. *Escherichia coli* cells overexpressing SoxR (17 kDa) were grown in the absence of PQ and purifications were performed as described in Materials and methods with (lanes 2−5) or without (lanes 6−9) 1 mM βME in the buffers. Samples of the purification fractions were analyzed by electrophoresis on an SDS−15% polyacrylamide gel followed by silver staining. The position and mass (kDa) of molecular weight markers (lane 1) are given on the left. Crude extracts, lanes 2 and 6; DE52 flow-through, lanes 3 and 7; heparin−agarose pool, lanes 4 and 8; DNA−affinity pool, lanes 5 and 9.

Table I. Purification of SoxR

Steps	−βME		+βME	
	Binding activity[a] (total U)	Total protein[b] (mg)	Binding activity[a] (total U)	Total protein[b] (mg)
I Crude extract	3.2×10^7	157.5	5.0×10^6	165.0
II DE52	3.0×10^7	130.0	5.0×10^6	132.6
III Heparin−agarose	1.2×10^7	4.8	1.9×10^6	5.2
IV DNA affinity	2.0×10^6	1.3	2.5×10^5	0.17

SoxR purification as described in Materials and methods was performed in the absence (−βME) or presence (+βME) of 2-mercaptoethanol in the purification buffers. The lower amount of binding activity in the crude extract containing βME was a result of lower SoxR expression in the cells before extraction; in other experiments, the same amount of activity was extracted initially and purified in the same percent yield.

[a]One unit (U) of binding activity specific for the *soxS* promoter is the amount of protein needed to shift 50% of the DNA into protein−DNA complex under standard assay conditions.

[b]Determined by Coomassie blue binding assay (Bradford, 1976) with BSA as standard.

Various alternative purification procedures were also tried. Cation exchange resins (phosphocellulose; BioRex 70) bound SoxR and allowed its recovery with a good yield, but did not improve the purity of fraction III. We were unable to identify conditions under which significant amounts of SoxR would bind or elute from affinity resins such as Cibacron blue−Sepharose or Procion red−Sepharose. Hydrophobic chromatography on phenyl−agarose allowed some purification, but with a significant loss of activity. Gel filtration chromatography on Ultrogel Aca54 or an FPLC Superose column led to substantial losses of SoxR, evidently a result of aggregation. This latter effect correlates with the poor solubility of SoxR under our conditions (≤200 μg/ml at pH 7.6 in 500 mM NaCl).

Characterizaton and metal content of SoxR

Fraction III SoxR protein purified in thiol-free buffers consistently exhibited a red−brown color that was absent

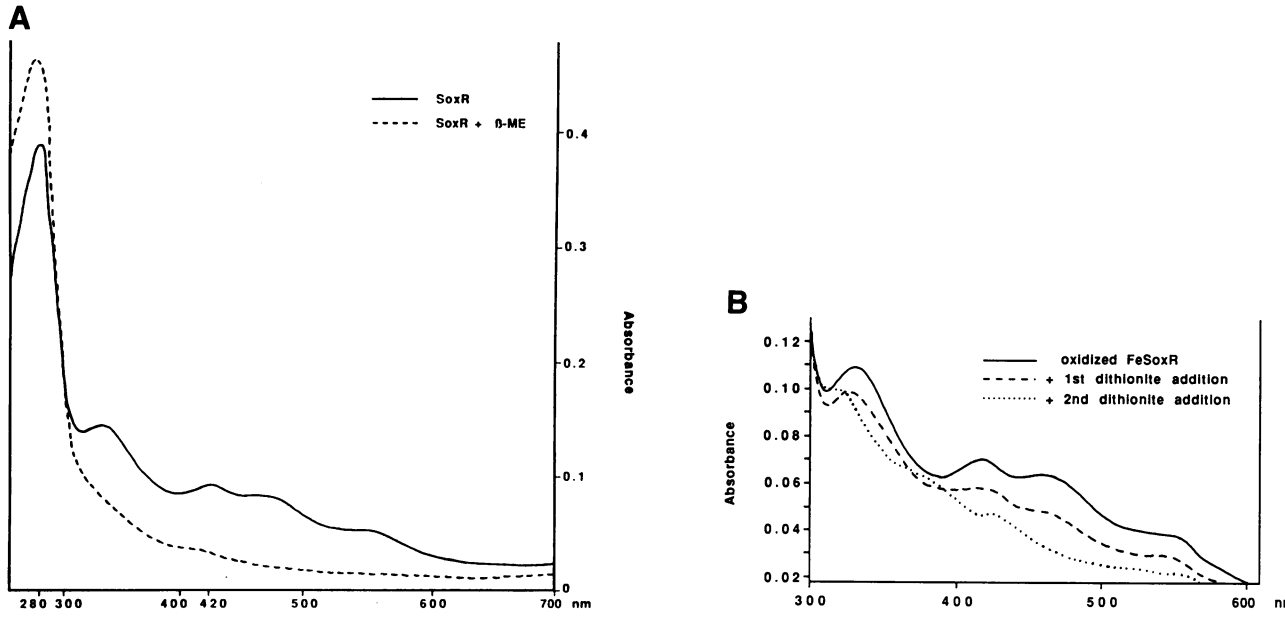

Fig. 2. Absorbance spectra of SoxR proteins. (**A**) Samples were purified in buffers without (solid line) or with 1 mM βME (dashed line). The data were collected on a Perkin-Elmer Lambda 3A spectrophotometer. (**B**) Dithionite reduction of Fe-SoxR. Fraction III Fe-SoxR (6.7 nmol in 1 ml) was scanned before (solid line) and after the anaerobic addition of 15.6 (dashed line) and 31.2 (dotted line) nanoequivalents of sodium dithionite. The spectra were recorded ≤45 s after adding the dithionite.

from protein samples purified in the presence of βME. This color difference is depicted in the UV/visible spectra of fraction III SoxR proteins shown in Figure 2A. In addition to the strong absorbance peak at 280 nm typical of most proteins, SoxR isolated in thiol-free buffers displayed four additional absorption maxima at 332, 414, 462 and 548 nm (Figure 2A). The absorbance of visible light by SoxR was affected only by βME in the purification buffers, and not by the treatment of the bacteria with PQ prior to extraction (data not shown).

The absorbance profile of the colored SoxR is similar to a number of proteins that contain iron–sulfur (FeS) centers (Holm and Ibers, 1977). We examined whether iron was present in our SoxR preparations using inductive coil plasma emission spectrometry. Among the 20 metallic elements examined in this way, only Fe was consistently present at significant levels in SoxR samples with visible absorbance and directly correlated with the amount of SoxR protein (Table II). In contrast, βME-exposed SoxR samples lacked most or all detectable iron, as well as the 19 other metals examined (Table II). An accurate stoichiometry of Fe in SoxR was established by using amino acid analysis for accurate measurement of the protein concentration. These measurements revealed that the amount of SoxR is overestimated by a factor of ~4 in the dye binding assay (Bradford, 1976) using BSA as the standard. This analysis revealed a maximum stoichiometry in these samples of Fe:SoxR = 1.6:1 (Table II). The low solubility of SoxR mentioned above precluded a determination of the content of inorganic sulfur in these experiments.

The redox activity of the SoxR FeS cluster was demonstrated by performing dithionite reductions of fraction III Fe-containing samples (Figure 2B). After addition of only ~5 equivalents of sodium dithionite per equivalent of SoxR monomer, the absorption of SoxR at 332, 414, 462 and 548 nm was diminished, while two new absorption maxima at 322 (probably due to the reduced dithionite) and 425 nm appeared (Figure 2B). We were unable to isolate the reduced form of Fe-SoxR in these experiments, because subsequent exposure to air caused rapid reoxidation of the protein to the form seen before dithionite treatment. These results suggest a significant oxidant sensitivity of Fe-SoxR, in line with the protein's proposed redox sensing role.

Table II. Metal content of partially purified SoxR

	SoxR samples				
	−βME			+βME	
	+PQ	−PQ	−PQ	+PQ	−PQ
Preparation	A	B	C	D	E
SoxR[a]	2.2	3.2	4.5	5.6	3.5
Fe	1.6	3.5	7.0	<0.9	<0.9
Ca	3.0	41.1	3.9	1.5	23.1
Mg	1.0	0.6	0.7	0.6	0.9
Cu	3.2	<0.8	<0.8	<0.8	<0.8
Sr	0.1	0.2	0.1	0.1	<0.1
Fe/SoxR	0.7	1.1	1.6	0.04	ND

All SoxR and metal concentrations are given in μM.
Five different SoxR samples (A: fraction IV; B–E: fraction III), purified from cells grown with (+PQ) or without (−PQ) paraquat and in buffers with (+βME) or without (−βME) 2-mercaptoethanol were analyzed with an ICP plasma emission spectrometer for 20 metallic elements. Data are shown only for those metals present at a detectable level in at least one protein sample. The 'less than' values indicate the limit of detection for that metal. The metals not detected at significant levels in any sample were: aluminum, barium, boron, cadmium, chromium, cobalt, lead, manganese, molybdenum, nickel, phosphorus, potassium, silicon, sodium and zinc.
[a]SoxR protein concentration was initially standardized by amino acid composition analysis and routinely estimated by densitometry of silver stained gels including a SoxR standard.

Binding of the soxS promoter by purified SoxR

We investigated whether the presence of Fe in purified SoxR affected the previously demonstrated ability of the protein in crude extracts to bind its DNA target, the *soxS* promoter

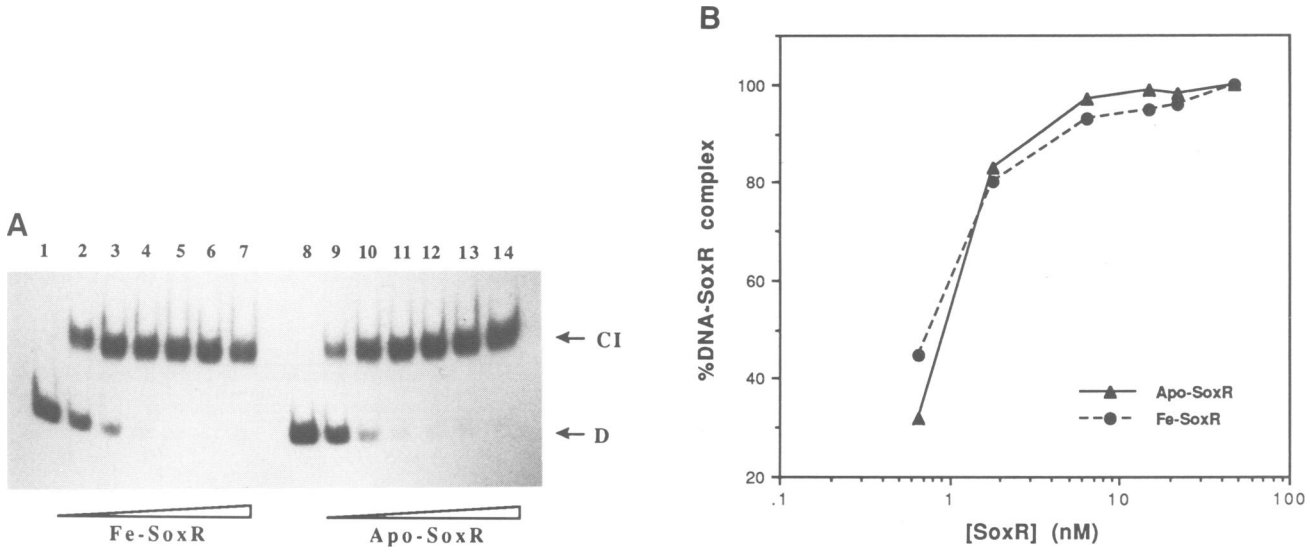

Fig. 3. DNA binding by SoxR protein. (**A**) Band shift assays were performed as described in Materials and methods by incubating a ^{32}P-labeled PCR fragment containing the *soxS* promoter with increasing amounts of fraction IV Fe-SoxR or apo-SoxR from PQ treated cells. The same degree of DNA binding was observed with Fe-SoxR and apo-SoxR from cells not treated with PQ (data not shown). DNA−protein complexes (CI, upper arrow) were resolved from free DNA (D, lower arrow) by electrophoresis in a polyacrylamide gel (Nunoshiba *et al.*, 1992). Lanes 1 and 8: no added protein; lanes 2 and 9: 0.65 nM of SoxR; lanes 3 and 10: 1.8 nM; lanes 4 and 11: 6.5 nM; lanes 5 and 12: 14.8 nM; lanes 6 and 13: 22.2 nM; and lanes 7 and 14: 47 nM. Similar experiments with three different Fe-SoxR and apo-SoxR samples were performed and yielded essentially the same result. (**B**) Quantitation of DNA binding by SoxR. The gel shown in (A) was analyzed by scanning densitometry with a BioImage system (Millipore). Fe-SoxR (filled circles, dashed line); apo-SoxR (filled triangles, solid lines).

(Nunoshiba *et al.*, 1992). The specific binding of SoxR to a DNA fragment containing the *soxS* promoter was determined using a gel mobility shift assay. Both purified Fe-containing and apo-SoxR formed a complex of the same apparent mobility (Figure 3A), and Fe-SoxR and apo-SoxR bound the *soxS* promoter with equal affinities (Figure 3B). Apparent dissociation constants (K_D) were estimated from these data as the concentration of SoxR that binds 50% of the total DNA probe in a standard band shift assay (Table III). These constants were calculated based on the demonstration that both Fe-SoxR and apo-SoxR are dimers even in dilute solution, as determined by sedimentation analysis (T.M.Bradley, E.Hidalgo and B.Demple, unpublished results). No difference in binding by Fe-SoxR compared with apo-SoxR was detected with five independent *soxS* DNA samples. We conclude that neither the presence of Fe, nor the treatment of the cells with PQ prior to extraction of the protein, affects the affinity of SoxR for its DNA binding site.

The general structure of the SoxR−DNA complex was also unaffected by Fe, as determined by DNase I footprinting. Samples of fraction IV were bound to the *soxS* promoter fragment and DNase I digestion performed as described in Materials and methods. Consistent with previous results with SoxR-containing crude extracts (Nunoshiba *et al.*, 1992), footprinting of either the transcribed or the non-transcribed strand (Figure 4A) revealed protection by SoxR of an ~30 nucleotide region spanning the −10 and −35 segments of the *soxS* promoter (Figure 4B). Several DNase hypersensitive sites were detected by this analysis for both strands (Figure 4A). One of these sites (at −28 on the non-transcribed strand; Figure 4A) was not noticed in previous experiments with crude extracts. Such a site could have been masked by other proteins present in the extracts or may have resulted from more limiting amounts of SoxR protein in those experiments. Most notably, there was no apparent difference in the extent of the SoxR footprint between Fe-SoxR and apo-SoxR (compare lanes 2 and 5 with lanes 3 and 6 in Figure 4A). However, cleavage at all of the DNase hypersensitive sites was consistently enhanced with apo-SoxR relative to Fe-SoxR (Figure 4A and data not shown).

Table III. Dissociation constants of SoxR and RNAP for the *soxS* promoter

Protein	K_D (M)
Apo-SoxR[a]	3.8×10^{-10}
Fe-SoxR	4.5×10^{-10}
RNAP[b]	4.0×10^{-8}
RNAP + Apo-SoxR	4.1×10^{-8}
RNAP + Fe-SoxR	2.4×10^{-8}

[a]K_D of purified apo-SoxR and Fe-SoxR samples (fraction IV) from PQ-treated cells was determined in parallel on the same DNA preparation. The estimation assumes that SoxR is a dimer (see text).
[b]K_D of RNAP with or without SoxR protein (samples as above) was determined in parallel on the same DNA preparation.

RNA polymerase binding

Gene activators can stimulate transcription by recruiting RNA polymerase (RNAP) to target promoters (Reznikoff *et al.*, 1985). We studied whether the affinity of *E.coli* RNAP (containing σ^{70}) for the *soxS* promoter was altered by the prior binding of either form of SoxR protein. Gel mobility shift assays were performed using the same DNA fragment with and without an initial incubation with saturating amounts of Fe-SoxR or apo-SoxR, followed by the addition of increasing amounts of purified RNAP (Figure 5A). RNAP formed complexes of much more strongly decreased mobility (CII in Figure 5A) than those formed by SoxR alone (CI in Figures 3A and 5A). In the SoxR-containing samples, CI was progressively converted to CII

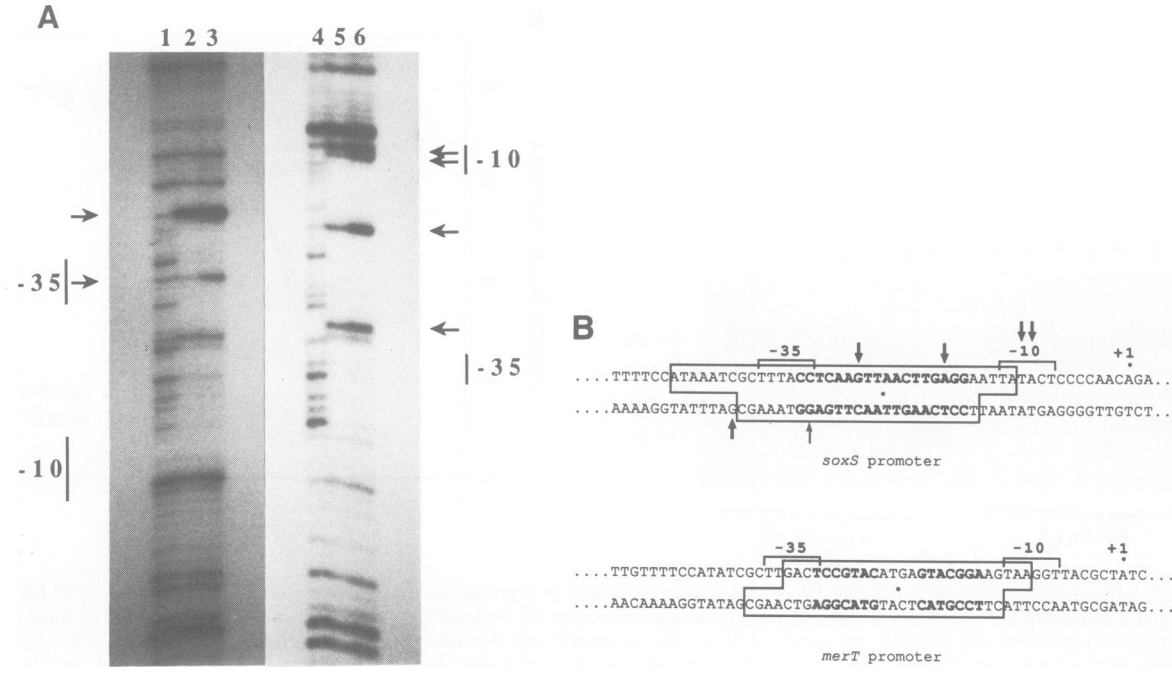

Fig. 4. DNase I protection patterns of the *soxS* promoter by SoxR. (**A**) A 180 bp fragment containing the *soxS* promoter was PCR labeled in the transcribed (lanes 1−3) or the non-transcribed (lanes 4−6) strand as described previously (Nunoshiba *et al.*, 1992). After incubating the DNA with protein extracts containing no SoxR protein as a negative control (lanes 1 and 4), 30 μg of fraction IV Fe-SoxR (lanes 2 and 5) or 30 μg of fraction IV apo-SoxR (lanes 3 and 6), DNase buffer and DNase I were added as described in Materials and methods. The −10 and −35 regions of the *soxS* promoter are indicated by vertical bars. The DNase hypersensitive sites, of different intensity for the Fe-containing and the apo-SoxR, are marked by arrows. (**B**) Protection data and comparison with the MerR binding site. The respective −10 and −35 boxes are indicated by brackets. The regions of dyad symmetry are in bold, and the middle of the repeat is indicated in each case with a dot. The transcriptional start points are marked +1. The boxes indicate the extent of DNase I protection by SoxR on the *soxS* promoter and by MerR on the *merT* promoter (O'Halloran *et al.*, 1989). The DNase hypersensitive sites are indicated by arrows, with the smaller arrow corresponding to a weaker site.

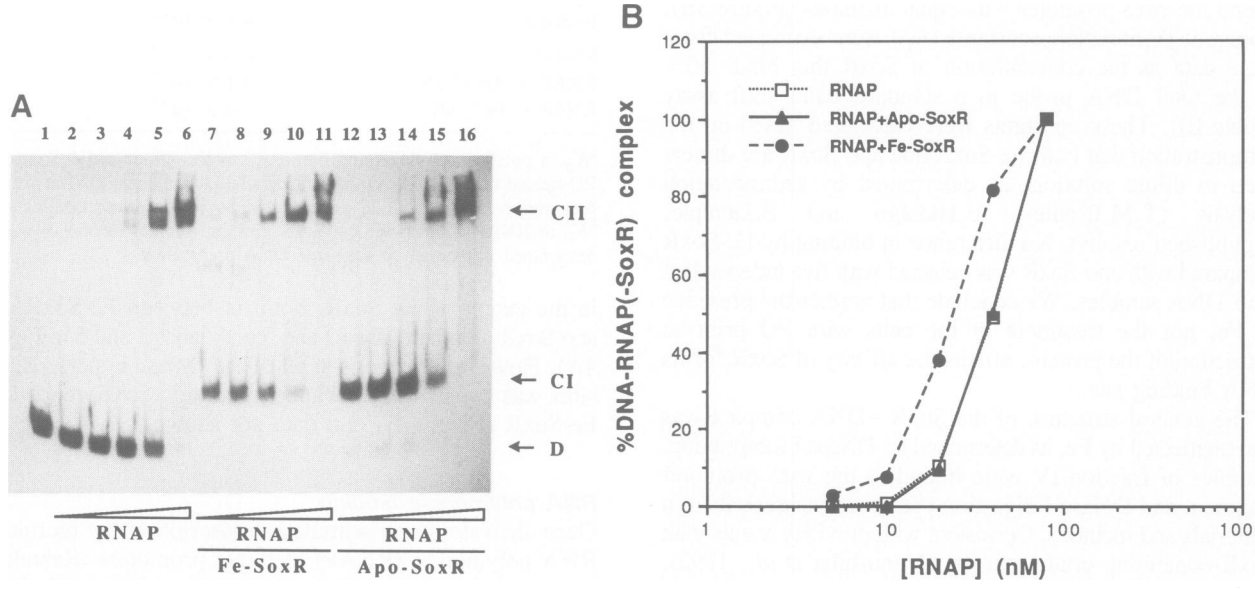

Fig. 5. DNA binding by RNAP in the presence or absence of SoxR. (**A**) A ^{32}P-labeled fragment containing the *soxS* promoter was incubated without (lanes 2−6) or with 15 nM fraction IV Fe-SoxR (lanes 7−11) or 15 nM fraction IV apo-SoxR (lanes 12−16). RNAP was then added at the following concentrations: lanes 2, 7 and 12: 5 nM; lanes 3, 8 and 13: 10 nM; lanes 4, 9 and 14: 20 nM; lanes 5, 10 and 15: 40 nM; and lanes 6, 11 and 16: 80 nM. Lane 1 shows a control of DNA incubated without proteins. Free DNA (D), DNA−SoxR complexes (CI) and DNA−RNAP complexes (CII, perhaps also containing SoxR) are indicated with arrows. (**B**) Binding isotherms for RNAP. The percentages of DNA−RNAP complexes, as determined by densitometry, were plotted versus log[RNAP] for RNAP alone (open squares, dotted line), RNAP plus Fe-SoxR (filled circles, dashed line) and RNAP plus apo-SoxR (filled triangles, solid line).

with increasing RNAP concentration (Figure 5A). Strongly retarded DNA−protein complexes (also labeled CII in Figure 5A) were also formed by RNAP in the absence of SoxR.

After quantitation of the formation of complexes CII in these experiments (Figure 5B), K_D values of RNAP for the *soxS* promoter were estimated as described above (Table III).

Using four independent *soxS* DNA preparations, the apparent binding affinity of RNAP for the *soxS* promoter was not significantly affected when apo-SoxR was first bound to the DNA compared with uncomplexed *soxS* DNA. Iron-containing SoxR protein consistently slightly improved the apparent affinity of RNAP for the *soxS* promoter, decreasing the estimated K_D by ~2-fold (Table III).

In vitro activation of the soxS promoter by SoxR: abortive transcription

Although the SoxR dependent induction of *soxS in vivo* can reach >50-fold, the above data show that this stimulation is not accounted for by the slight enhancement of RNAP binding by Fe-SoxR *in vitro*. We therefore checked whether either form of SoxR stimulates initiation of transcription by *E.coli* RNAP at the *soxS* promoter *in vitro*. Initiation was followed by the formation of the abortive product ApGpApU from an ApG dinucleotide initiator, ATP and [α-^{32}P]UTP (Figure 6A). In the absence of SoxR protein, only a faint product was detected (Figure 6B). The synthesis of the abortive product was increased substantially by preincubation with Fe-SoxR, and this was unaffected by PQ treatment of the cells from which SoxR was extracted (lanes 2 and 3 in Figure 6B). In contrast, the apo-SoxR samples (with or without PQ treatment of the cells) gave no detectable increase in abortive initiation (lanes 4 and 5 in Figure 6B).

A quantitative estimate of the difference in abortive transcription as a function of the amount of Fe-SoxR or apo-SoxR was made by examining a range of SoxR concentrations (1.5−600 nM). In these experiments, the activation of transcription by Fe-SoxR increased with the concentration of SoxR protein in the transcription reaction (Figure 6C). Even at the highest concentrations, apo-SoxR was without a detectable stimulatory effect. Since significant stimulation of the abortive initiation was produced by 7.5 nM Fe-SoxR (lane 3) and none at all by 600 nM apo-SoxR, activated SoxR stimulated transcription ≥80-fold compared with the inactive form. This magnitude of effect approximated the maximum transcriptional activation measured for SoxR *in vivo* (Nunoshiba *et al.*, 1992; Wu and Weiss, 1992; Nunoshiba and Demple, 1993) and implies that no components other than activated SoxR are needed for this stimulation.

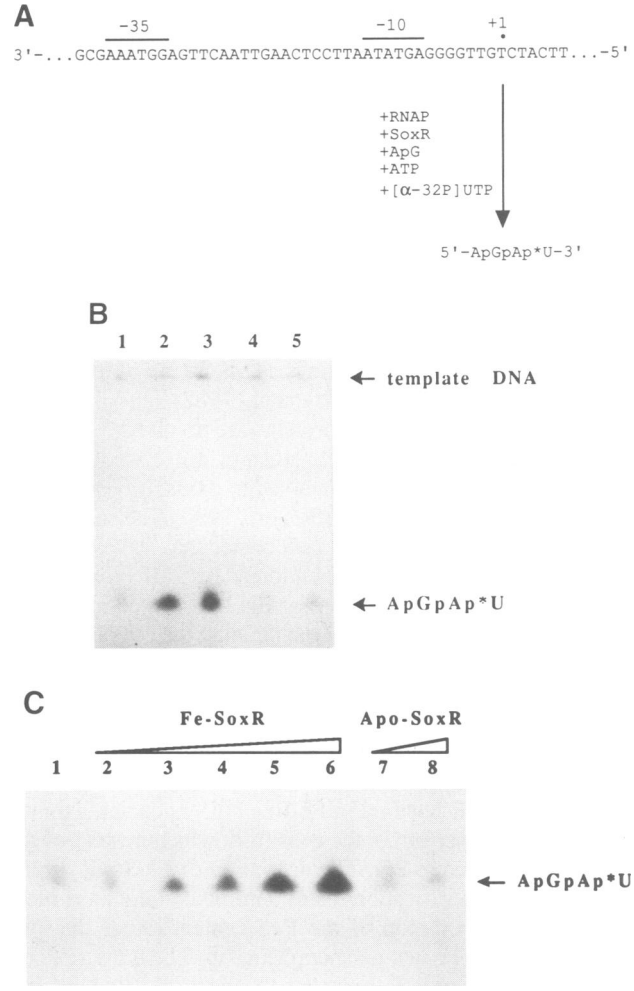

Fig. 6. Abortive initiation at the *soxS* promoter *in vitro* with purified SoxR. (**A**) Scheme for abortive transcription from the *soxS* promoter. The template strand is shown, and the −10, −35 and +1 positions indicated. In the presence of RNAP and SoxR, transcription initiates at *soxS* with an ApG dinucleotide. The further addition of ATP and [α-^{32}P]UTP limited the elongation of the nascent transcript to a tetranucleotide (5′-AGAU-3′). (**B**) Abortive transcription with different SoxR samples. RNAP (5 nM) was added in all samples. Lane 1, no SoxR added. Lanes 2−5, fraction III SoxR protein (added to a final concentration of 300 nM) from four different preparations: cells were grown with PQ for the proteins added in lanes 2 and 4; βME was added in the purification buffers for the proteins of lanes 3 and 4. The template DNA and the abortive product are indicated by arrows. (**C**) Concentration dependence of *soxS* transcriptional activation by SoxR. RNAP (5 nM) was added in all samples. Fraction III Fe-SoxR (lanes 2−6) or apo-SoxR (lanes 7 and 8) were incubated with the fragment containing the *soxS* promoter prior to addition of RNAP. Lane 1: no SoxR protein added; lanes 1 and 7: 1.5 nM SoxR; lane 3: 7.5 nM SoxR; lane 4: 30 nM SoxR; lane 5: 150 nM SoxR; lanes 6 and 8: 600 nM SoxR.

Discussion

SoxR protein controls the initial step of a redox sensitive superoxide stress pathway by activating the transcription of a single gene, *soxS* (Demple and Amábile-Cuevas, 1991; Nunoshiba *et al.*, 1992; Wu and Weiss, 1992). We have now shown that SoxR is a novel transcription factor containing non-heme iron that can activate initiation by RNAP *in vitro* up to 100-fold. This FeS center is not required to maintain SoxR protein structure, because the apoprotein binds its DNA target with the same affinity as Fe-SoxR. Since only Fe-SoxR stimulates transcriptional initiation, the role of the FeS center must therefore be to modulate gene activation by SoxR.

Biological evidence implicates SoxR as the redox sensing component of the two stage pathway of sequential gene activation in the *soxRS* regulon (Demple and Amábile-Cuevas, 1991; Nunoshiba *et al.*, 1992; Wu and Weiss, 1992). In this connection, the nature of the SoxR FeS center is of great interest because of the numerous oxidation/reduction reactions associated with iron−sulfur proteins (Beinert, 1990).

Aerobically purified SoxR samples showed a maximum Fe:SoxR ratio of 1.6:1. Such a ratio may underestimate the full complement of Fe in SoxR intracellularly, since many iron−sulfur centers are labile and subject to loss during purification (Beinert, 1990). If the actual ratio is two Fe per subunit, this could reflect the presence of two Fe_2S_2 centers or one Fe_4S_4 cluster per SoxR dimer; two Fe_2S_2 centers could be located as either one per subunit or both between subunits. An iron−sulfur center liganded to two protein subunits has been described for the nitrogenase from

Azotobacter vinelandii (Georgiadis *et al.*, 1992). Furthermore, the dimeric MerR protein, a transcriptional activator related to SoxR in both structure and function (Amábile-Cuevas and Demple, 1992; this work), binds Hg^{2+} with two cysteines of one subunit and a different cysteine from the other subunit of the homodimer (Hellman *et al.*, 1990). UV−visible spectroscopy does not readily distinguish between the different possible types of FeS clusters, and the possibility of a one-iron center in each subunit has not been ruled out. Additional biophysical approaches, such as electron paramagnetic resonance, electron nuclear double resonance spectroscopy or Mössbauer spectroscopy will be required to ascertain more precisely the nature of the cluster (Orme-Johnson and Orme-Johnson, 1982).

Most known iron−sulfur proteins are involved in electron transfer, although some FeS clusters are constituents of enzyme active sites but not involved in oxidation−reduction processes, such as aconitase (Beinert, 1990; Klausner *et al.*, 1993). In other proteins, the FeS center may have a strictly structural role, as in *E.coli* endonuclease III (Kuo *et al.*, 1992). In SoxR, the FeS center is likely involved in sensing superoxide stress, rather than maintaining the overall protein structure. Several observations indicate that the oxidation state of the SoxR FeS cluster, rather than its presence or absence, controls the biological activity of the protein. (i) The SoxR FeS center is redox active, with the air-oxidized protein sensitive to reduction by dithionite, as we have shown. (ii) SoxR purified in Fe-free buffers contains tightly bound Fe, whether or not the cells from which it was isolated had been exposed to PQ. The activation of Fe-SoxR during extraction and purification under aerobic conditions is more likely due to oxidation of the FeS center, rather than the reconstitution of Fe into an apoprotein. (iii) The transcriptional activity of Fe-SoxR is resistant to chelators (unpublished data), indicating that the metal is tightly bound, although Fe is releasable by continuous exposure to thiols during the purification. (iv) FeS centers in various dehydratases are very sensitive targets for oxidative inactivation by $O_2^{\cdot -}$ specifically (Gardner and Fridovich, 1991). Evolution could have taken advantage of such a reaction in order to generate a protein−FeS sensor of $O_2^{\cdot -}$ stress (or of $NO^{\cdot}$) to activate gene expression.

How might the FeS center of SoxR act as a redox sensor for transcriptional activation? As argued above, the key for activation of SoxR during $O_2^{\cdot -}$ stress is probably a redox reaction involving a pre-existing FeS center in the protein. Since transcriptionally active SoxR has an FeS center that can be chemically reduced, activated Fe-SoxR is evidently in the oxidized state. Therefore, reduced (non-activated) Fe-SoxR would be oxidized by $O_2^{\cdot -}$ or some signal intimately connected with superoxide generation (Nunoshiba, 1992) or perhaps by exposure to nitric oxide (Nunoshiba *et al.*, 1993). $NO^{\cdot}$ could also displace one or more cysteine ligands of the FeS center (Drapier *et al.*, 1991) to activate SoxR. Given the apparent sensitivity of Fe-SoxR to oxidation, this hypothesis predicts that the protein is maintained in the reduced state in *E.coli* even during vigorous aerobic growth (Nunoshiba *et al.*, 1992). Active reduction of the FeS protein by an NADPH dependent reductase could account for the sensitization to *soxRS* induction in strains lacking glucose-6-phosphate dehydrogenase (Liochev and Fridovich, 1992).

Mechanisms that might couple oxidation (or reduction) of an FeS center to a protein conformational change have not been defined. In principle, a rearrangement of the protein structure could take place through the breakage and creation of different bonds, or through a redox generated change in specific coordination geometry. In the case of nitrogenase, ATP hydrolysis at a distant intersubunit active site leads to allosteric changes that affect the redox potential of its FeS center, also located at the subunit interface (Georgiadis *et al.*, 1992). The details of this allostery are as yet unknown. The redox modification of an FeS center could also be transferred to an amino acid in the polypeptide chain, such as the Fe dependent tyrosyl radical of *E.coli* ribonucleotide reductase (Mulliez *et al.*, 1993). The possibility that Fe replaces another metal is unlikely, because significant amounts of 19 other metals were not reproducibly found in either active or inactive SoxR.

The mechanism by which activated SoxR triggers transcription must be unusual. SoxR binding overlaps the RNAP binding region of the *soxS* promoter rather than an upstream site. Our data are consistent with the interpretation that Fe-SoxR activates transcription by distorting DNA structure rather through SoxR−RNAP protein interactions. For example, activated Fe-SoxR enhances the binding affinity of RNAP for the *soxS* promoter by only 2-fold but clearly diminishes cleavage at some DNase hypersensitive sites compared with non-activated SoxR. Such effects are reminiscent of the homologous MerR protein, which in the Hg^{2+} activated form strongly stimulates open complex formation and initiation by RNAP, without any large effect on binding (O'Halloran *et al.*, 1989; Ansari *et al.*, 1992). For Hg-MerR, this activation depends on the suboptimal 19 bp spacing between the −10 and −35 promoter sites (Parkhill and Brown, 1990), which the activated protein is proposed to bring into better helical alignment by means of a specific untwisting effect (Ansari *et al.*, 1992). An analogous 19 bp spacing can readily be drawn for the *soxS* promoter (Figure 4B), by utilizing a probable −10 site 2 bp closer to the transcription start than proposed earlier (Amábile-Cuevas and Demple, 1991; Wu and Weiss, 1991). This promoter might respond to torsional activation by Fe-SoxR.

We do not know whether counterparts to SoxR exist in other organisms. Unrelated regulatory proteins are known that may sense specific oxidative signals and activate transcription. OxyR protein was proposed to employ a specific cysteine residue to sense H_2O_2 stress and activate its target genes (Storz *et al.*, 1990). Fnr has been postulated to sense O_2 availability through an FeS cluster, but Fe-containing Fnr has not been isolated without the addition of iron in the purification buffers (Green *et al.*, 1991). In eukaryotes, IRE-BP probably responds to iron availability, rather than to a redox signal (Klausner *et al.*, 1993). The same can be said for its bacterial counterpart, Fur (Bagg and Neiland, 1987). In eukaryotic cells, reactive oxygen intermediates apparently activate NF-$\varkappa$B, a transcriptional regulator of genes involved in inflammatory and acute phase responses (Meyer *et al.*, 1993), although neither NF-$\varkappa$B nor its companion I-$\varkappa$B seem to be the direct sensors of oxidation (Schreck *et al.*, 1992). Both oxidants (Devary *et al.*, 1992) and reductants (Meyer *et al.*, 1993) seem to activate DNA binding by Jun/Fos. As for NF-$\varkappa$B, the redox sensing component for Jun/Fos activation apparently resides upstream in the signalling cascade (Meyer *et al.*, 1993). SoxR seems

to be the first redox sensitive transcriptional regulator found that contains a tightly bound FeS center needed, not for structural purposes, but for its transcriptional activator role.

Materials and methods

Purification of SoxR

Strain XA90 [Δ(*lac pro*)*XIII ara nalA argE* (Am) *thi* Rif^r (F′ *lacI^{q}1ZY proAB*)], from M.Ptashne via G.Verdine, was transformed with the SoxR expression plasmid (pKOXR), described previously (Nunoshiba *et al.*, 1992). An overnight culture of XA90/pKOXR grown in LB-ampicillin medium (100 μg ampicillin per ml of LB medium; Miller, 1992) was diluted 100-fold into 1 l of fresh medium in a 4 l flask and shaken at 37°C until the culture reached an optical density of 0.5 at 600 nm. Isopropyl-β-thio-D-galactoside (IPTG) was then added to a final concentration of 0.5 mM and shaking continued at 37°C for 2.5 h. Where indicated, PQ was added to the culture to a final concentration of 1 mM for the final 45 min. The cells were then harvested, washed with M9 salts (Miller, 1992) and resuspended in 20 ml of buffer A [50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.6, 0.1 M NaCl]. In some preparations, 1 mM βME was present in the buffers. The cells were broken by three passes through a French pressure cell at 9000–10 000 p.s.i. Debris and unbroken cells were then removed by centrifugation at 30 000 *g* for 45 min. The cleared cell extract (fraction I) was applied to a 1 × 5 cm column of DE52–Sephadex (Whatman) equilibrated with buffer A. The SoxR protein, monitored by SDS–PAGE and its DNA binding activity to the *soxS* promoter (Nunoshiba *et al.*, 1992; see below) flowed through the column. This flow-through (fraction II) was applied to a 25 ml column of heparin–agarose (BRL). The column was then washed with 100 ml of buffer A and 100 ml of 50 mM HEPES, pH 7.6, 0.35 M NaCl. The protein was eluted with 100 ml of 50 mM HEPES, pH 7.6, 0.5 M NaCl. Active fractions were pooled (fraction III) and diluted 5-fold with 50 mM HEPES, pH 7.6, to a final NaCl concentration of 0.1 M. The diluted sample was then applied to a *soxS* promoter–DNA affinity column (see below) equilibrated with the same buffer. The column was then washed with 20 ml buffer A and SoxR eluted with a gradient of 0.1–1.2 M of NaCl in 40 ml 50 mM HEPES, pH 7.6. The fractions with the highest DNA binding activity were pooled and concentrated under stirring in an Amicon ultrafiltration cell using a PM10 membrane (fraction IV).

Preparation of a SoxR-binding affinity column

A DNA affinity column containing the *soxS* promoter was prepared according to Larson and Verdine (1992). Two synthetic 39mer oligonucleotides were employed. These had the sequences 5′-TTGAAGTATAATTCCTCAAGTTAACTTGAGGTAAAGCGA-3′ and 5′-TXAATCGCTTTACCTCAAGTTAACTTGAGGAATTATACT-3′, X being 4-*O*-(2,4,6-trimethylphenyl)-2′-deoxyuridine phosphoramidite (MacMillan and Verdine, 1990), which pairs as C and contains a primary amino group linked through a six-carbon chain to the base. After purification, annealing and ligation, the double-stranded oligonucleotide was covalently coupled to NHS–HiTrap columns (Pharmacia) according to the manufacturer's instructions.

Protein and metal analysis

Protein concentrations during purification were determined by the Bradford method (1976), using BSA as a standard. Different purification fractions of SoxR were electrophoresed in 15% polyacrylamide gels containing SDS and stained with Coomassie blue (Hames, 1987) or silver (Poehling and Neuhoff, 1981). The purity and content of SoxR in the different protein preparations was then determined by scanning densitometry of the stained gels using a BioImage system (Millipore). For purified SoxR, the protein concentration was also determined by amino acid analysis; hydrolysis of the sample was done by standard vapor phase hydrolysis using a Waters Pico-tag work station. The hydrolysate was then transferred to an ABI 420 Derivatizer for conversion to PTC-amino acids, which were then injected onto an ABI 130 online HPLC for analysis. This analysis was performed at the Molecular Biology Core Facility at the Dana-Farber Cancer Institute (Boston, MA). Samples analyzed in this way were then used as standards to calculate the absolute amounts of SoxR protein in silver stained SDS–polyacrylamide gels.

The metal content of purified SoxR was analyzed by inductive coil plasma emission spectrometry with a Jarrell-Ash 965 ICP. This analysis was carried out by the Chemical Analysis Laboratory, Institute of Ecology, University of Georgia (Athens, GA).

In vitro reduction of SoxR

Sodium dithionite (Sigma) solutions were prepared by dissolving 1–2 mg of dithionite in previously degassed 1 ml of 10 mM HEPES, pH 7.6. Solutions were titrated with flavin adenine dinucleotide (FAD; Sigma) as a standard. Either FAD or SoxR reductions were performed under anaerobic conditions in a sealed cuvette. Spectra of oxidized and reduced forms were recorded with a Hewlett-Packard model 8452A spectrophotometer (courtesy of C.T.Walsh).

DNA–protein binding

A 180 bp fragment containing the *soxS* promoter region was PCR amplified and ^{32}P-labeled as described previously (Nunoshiba *et al.*, 1992; Hidalgo *et al.*, 1993) and purified on a small Sephadex G50 column followed by electrophoresis in polyacrylamide gels (5%). Labeled DNA was quantified with a fluorometer (Hoefer, TKO 100) using pBR322 digested with *Hae*III as the standard. The DNA binding reaction mixtures (20 μl) contained 75 mM KCl, 2 mM dithiothreitol, 10% glycerol, 0.1 μg of poly(dI)-poly(dC), 2 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.5, 0.5–1.0 fmol of labeled DNA and the indicated amount of SoxR protein. After a 10 min incubation at 25°C, 1 μl of *E.coli* RNAP (Promega or Pharmacia) was added at various concentrations as indicated and the incubation continued at 37°C for 15 min. Electrophoresis and autoradiography were performed as described previously (Nunoshiba *et al.*, 1992; Hidalgo *et al.*, 1993). Because the presence of $MgCl_2$ in the binding reaction did not affect DNA binding by purified SoxR to the *soxS* promoter, we incorporated this metal salt in our standard reactions to allow subsequent RNAP binding to DNA. The DNA binding activity of SoxR was defined as follows: one unit of binding activity is the amount of protein needed to shift 50% of the DNA into the SoxR–DNA complex under the standard assay conditions (without RNAP).

DNase footprinting

DNase I protection experiments were performed by a modification of the method described previously (Nunoshiba *et al.*, 1992). After incubating the probe DNA with protein as described above, DNase I (Sigma) was added (1–20 ng in 1 μl of 10 mM Tris–HCl, pH 8.0, 2.5 mM $MgCl_2$, 75 mM KCl, 2 mM dithiothreitol, 20 mM $CaCl_2$ and 50 μg/ml BSA) and the reaction incubated at 25°C for 2 min. The reactions were stopped, the DNA precipitated and samples electrophoresed as described (Nunoshiba *et al.*, 1992).

Abortive transcription

For the standard assay, 10–50 fmol of unlabeled DNA was incubated in 20 μl with the indicated amounts of SoxR protein and RNAP as for the DNA–protein binding assay. Polymerization reactions were initiated by the addition of 10 μl containing 0.6 mM of the dinucleotide initiator ApG (Sigma), 0.1 mM ATP, 0.25 μM [α-^{32}P]UTP (>600 Ci/mmol, transcription grade; ICN), 300 μg/ml heparin, 0.3 M potassium glutamate, 30 mM Tris–HCl, pH 8.0, 3 mM $MgCl_2$, 0.3 mg/ml BSA, 3 mM $CaCl_2$, 15% glycerol and 3 mM dithiothreitol. After 15 min incubation at 37°C the reactions were stopped by the addition of 3 μl 60 mM EDTA, 30% glycerol and 5 μl of formamide loading buffer. Aliquots (4 μl) of these mixtures were then electrophoresed on a 20% denaturing polyacrylamide gel (Sambrook *et al.*, 1989) at 400 V for 2 h.

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