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Cysteine-to-alanine replacements in the *Escherichia coli* SoxR protein and the role of the [2Fe-2S] centers in transcriptional activation

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ABSTRACT

The *Escherichia coli* *soxRS* regulon activates oxidative stress and antibiotic resistance genes in two transcriptional stages. SoxR protein becomes activated in cells exposed to excess superoxide or nitric oxide and then stimulates transcription of the *soxS* gene, whose product in turn activates ≥ 10 regulon promoters. Purified SoxR protein is a homodimer containing a pair of [2Fe-2S] centers essential for *soxS* transcription *in vitro*. The [2Fe-2S] centers are thought to be anchored by a C-terminal cluster of four cysteine residues in SoxR. Here we analyze mutant SoxR derivatives with individual cysteines replaced by alanine residues (Cys→Ala). The mutant proteins in cell-free extracts bound the *soxS* promoter with wild-type affinity, but upon purification lacked Fe or detectable transcriptional activity for *soxS* *in vitro*. Electron paramagnetic resonance measurements *in vivo* indicated that the Cys→Ala proteins lacked the [2Fe-2S] centers seen for wild-type SoxR. The Cys→Ala mutant proteins failed to activate *soxS* expression *in vivo* in response to paraquat, a superoxide-generating agent. However, when expressed to <5% of the cell protein, the Cys→Ala derivatives increased basal *soxS* transcription 2–4-fold. Overexpression of the Cys119→Ala mutant protein strongly interfered with *soxS* activation by wild-type SoxR in response to paraquat. These studies demonstrate the essential role of the [2Fe-2S] centers for SoxR activation *in vivo*; the data may also indicate oxidant-independent mechanisms of transcriptional activation by SoxR.

INTRODUCTION

Bacteria regulate many genes in response to imbalances in the production and disposal of reactive oxygen species; such conditions are often called 'oxidative stress' (1). In *Escherichia coli*, a set of <12 dispersed promoters under the control of the *soxRS* locus is activated when cells are exposed to sublethal levels of compounds, such as paraquat (PQ) (2,3), which generate intracellular superoxide, or to nitric oxide (4,5). This *soxRS*

regulon controls antioxidant functions [e.g. superoxide dismutase or glucose-6-phosphate dehydrogenase (1)], repair of oxidative DNA damage [endonuclease IV (1)] and antibiotic resistance genes [*micF* (6) or *acrAB* (7)]. The *soxRS* system may also provide resistance to the toxicity of organic solvents and some heavy metals (8). The *soxRS* regulon is switched on in two transcriptional stages: existing SoxR protein is activated by an intracellular redox signal and triggers transcription of the *soxS* gene; the resulting SoxS protein binds and activates transcription from the various regulon promoters (1).

SoxR protein is the master regulator of the *soxRS* response (9,10). SoxR *in vitro* binds and strongly stimulates transcription of the *soxS* promoter by the exponential-phase RNA polymerase (RNAP) containing the 70 S protein (11). The activity of SoxR as a transcription factor is completely dependent on the presence of non-heme iron in the protein (11). The metal is present in the active homodimeric SoxR protein as a pair of [2Fe-2S] clusters, which are in the oxidized form when the protein is isolated from cells (12,13). SoxR activity can be regulated either by the assembly and disassembly of its iron-sulfur clusters (14,15) or by oxidation-reduction of the [2Fe-2S] centers, with the oxidized protein being the transcriptionally active form *in vitro* (16,17). Recent *in vivo* experiments support the interpretation that reduced SoxR is transcriptionally inactive (18).

From the foregoing it is clear that the [2Fe-2S] centers in SoxR are intimately involved in the protein's function as a transcription regulator *in vitro*, but their *in vivo* importance has not been validated. Attempts to control iron availability are complicated by the essential roles of Fe in other proteins, and by the complex regulation governing the assimilation and storage of this metal (19). Initial spectroscopic analysis indicates that each [2Fe-2S] center in SoxR is anchored by four thiol ligands, which accounts for the four cysteine residues present in each protein monomer (12,13). In this work we have tested the biological role of the [2Fe-2S] centers by mutating the individual cysteine residues of SoxR. These mutations eliminate the activation of SoxR by oxidative stress *in vivo* and prevent the assembly of stable iron-sulfur centers in the protein, but still allow a significant level of basal SoxR activity when the mutant proteins are expressed at high levels.

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MATERIALS AND METHODS

Strains and plasmids

This work employed the *E. coli* K-12 strains carrying single copy operon fusions present in lysogenized phage λ : strain TN521, a derivative of GC4468 (2) but Δ soxRS::zjc2205-kan λ (soxR⁺soxS2::lacZ) (20); and strain TN5311, as TN521 except λ (Δ soxR soxS2::lacZ) (20) and containing F2 proAB lacI^q lacZ Δ M15::Tn10 (21), transferred from XL1-blue (Stratagene, La Jolla, CA) by conjugation. The pBluescript plasmid was from Stratagene and does not contain an inducible promoter for recombinant gene expression. The plasmid pSE380 contains the lacI^q gene and the lac repressor-regulated trc promoter for controlled expression of recombinant genes (Invitrogen, San Diego, CA). The plasmid pKEN2 [a generous gift of G. Verdine, Harvard University (11)] contains a lac-regulated derivative of the tac promoter for controlled expression of recombinant genes but does not contain its own lacI gene; expression from pKEN2-derived plasmids is regulated by chromosomal lacI.

Construction of cysteine-to-alanine mutations

Four mutant soxR genes, each encoding an alanine residue substituting for a SoxR cysteine (residues 119, 122, 124 and 130), were constructed by using a plasmid containing the soxRS locus [pBD100; (22)] as a template for sequential rounds of polymerase chain reaction (PCR). For the first round of amplification, primers T3 (52-ATTAACCTCACTAAAG-32) and T7 (52-AATAC-GACTCACTATAG-32), specific for sequences in the pBluescript KS vector were used together with mutagenic primers converting the cysteine codon (TGT or TGC) to an alanine codon (GCT or GCC; underlined below).

- C119A 52-GAACTGGACGGAGCTATTTGGTTGTGG-32
- C119A 52-CCACAACCAATAGCTCCGTCAGTTC-32
- C122A 52-GATGTATTTGGTGCTGGCTGCCTTT-32
- C122A 52-AAAGGCAGCCAGCACCACAATACATC-32
- C124A 52-TGCGCGAAAGGGCGCCACAACCAA-32
- C124A 52-TTGGTTGTGCGCCCTTTCGCGCA-32
- C130A 52-CAGTGATGCCCGTTGCGTA-32
- C130A 52-TACGCAACGGGCCATCACTG-32

Overlapping PCR products were generated using the pairs of primers, pBD100 (10 ng) as the template, and 1 U cloned *Pfu* DNA polymerase (Stratagene) and isolated from the reaction mixtures using a commercially available spin column (Qiagen, Chatsworth, CA). Approximately 10 ng of each pair of overlapping soxR fragments were then combined with 50 pmol of the T3 and T7 primers in a 100 μ l reaction mixture. After denaturation and reannealing of the overlapping complementary cysteine coding regions, PCR yielded fragments (728 bp) containing the full-length mutated genes. These were purified and digested with *Eco*RI and *Hind*III (New England Biolabs, Beverly, MA), electrophoresed in a 1% agarose gel in Tris-acetate/EDTA buffer (23) and recovered from gel slices using DEAE membranes (Schleicher and Schuell, Keene, NH). The purified fragments were subcloned into *Eco*RI/*Hind*III-digested pBluescript and transformed into XL1-Blue cells. Transformants were selected on LB agar plates (21) containing 75 μ g/ml ampicillin, 15 μ g/ml tetracycline and the LacZ⁺ indicator 5-bromo-4-chloro-3-indolyl- β -galactopyranoside (21). White colonies were selected and analyzed by PCR using the T3 and T7 primers to verify the presence of

full-length soxR in the plasmids. Plasmid DNA was purified from positive clones and both strands of the soxR inserts completely sequenced by the dideoxy chain termination method (23) using Sequenase II (US Biochemical) and the following primers: T3, T7, SRKR (52-GGGCAACACGCCAAACGCT-32), SRKF (52-AGC-GTTTGGCGTCTTGCCC-32), E4 (52-GCGCGGATCCCAGC-GGCGATATAAA-32) and E5K (52-GCGCGAGCTCGCTTTCG-TCCCAATGG-32).

The four mutant cysteine sequences were subcloned from the pBluescript vectors into pKEN2, generating the plasmids pKENC119A, pKENC122A, pKENC124A and pKENC130A. The C119A mutant gene was also subcloned into pSE380 to generate pSEC119A for subsequent studies. Despite several attempts, stable insertion of the other cysteine mutant genes into pSE380 was unsuccessful.

Preparation of whole cell homogenates and partially purified protein

Plasmids derived from pKEN2 were transformed into *E. coli* strain TN5311 and grown in 200 ml LB broth (21) containing 75 μ g/ml ampicillin, 15 μ g/ml streptomycin and 15 μ g/ml kanamycin to OD₆₀₀ \pm 0.5–1. Isopropyl- β -D-thio-galactopyranoside (IPTG) was then added to a final concentration of 0.5 mM, and the incubation was continued for 120 min at 37°C. The cells were harvested by centrifugation at 10 000 g at 4°C, resuspended and washed three times with ice-cold M9 salts (21). The final cell pellet was resuspended to a volume of 1.0 ml with 50 mM HEPES–NaOH, pH 7.6, 0.1 M NaCl, and lysed by agitation with glass beads (400 μ l beads/ml) in a Mini-bead beater (Biospec Products, Bartlesville, OH) for 3 min. Following centrifugation at 10 000 g for 45 min at 4°C, the supernatants were collected and frozen at –200°C or –80°C until assay. Cell extracts were resolved on 15% SDS–polyacrylamide gels (24) and stained with Coomassie blue as previously described (11).

SoxR mutant proteins were also extensively purified using DE52–Sephacrose and heparin–agarose column chromatography as described previously (11).

Iron and protein determinations

The Fe content of partially purified SoxR proteins (11) was determined by inductively coupled plasma emission spectrometry at the Chemical Analysis Laboratory, Institute of Ecology, University of Georgia (Athens, GA). SoxR concentrations were estimated by scanning densitometry analysis (Visage System, Millipore, Milford, MA) of Coomassie blue-stained SoxR in SDS–polyacrylamide gels (24) using a SoxR standard previously quantified by amino acid analysis.

DNA–protein binding

The binding affinity of wild-type and mutant SoxR proteins for the soxS promoter *in vitro* was analyzed by electrophoretic mobility shift assays, as previously described (9,11).

Preparation of cell suspensions

TN5311 transformed with the expression plasmid pKEN2 or its soxR-containing derivatives pKOXR [containing the wild-type soxR gene; (22)], pKENC119A, pKENC122A, pKENC124A or pKENC130A were grown overnight in LB broth containing ampicillin (100 μ g/ml). A fresh 125-ml aliquot of the same

Cysteine substitution mutations in SoxR

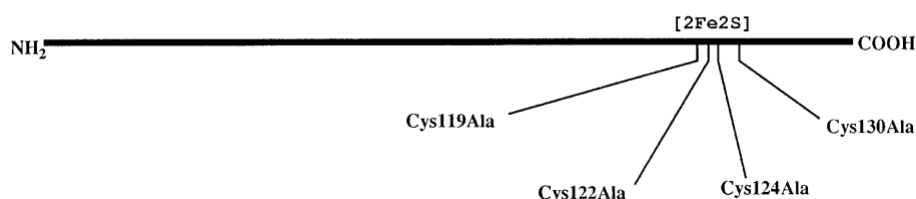


Figure 1. Location of [2Fe-2S] cluster and cysteine replacements in SoxR protein.

medium was inoculated with 1.25 ml of overnight culture and incubated at 37°C with shaking at 250 r.p.m. for 110 min. IPTG was added to a final concentration of 0.5 mM, and the incubation continued at 37°C with shaking for an additional 120 min. The cells were harvested by centrifugation and resuspended in 0.5 ml of 50 mM HEPES–NaOH, pH 7.6, 0.1 M NaCl. Freshly dissolved dithionite, 0.1 M dithionite in 1 M HEPES–NaOH pH 7.6, was then added to the cell paste to a final concentration of 1 mM. Aliquots (300–400 μ l cell paste) were immediately 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 Western blot analysis of samples lysed directly in sample buffer (24), using previously quantified SoxR as a standard.

Immunological methods

Wild-type *E. coli* SoxR protein was purified to near homogeneity as described previously (11). The purified protein was emulsified in complete Freund's adjuvant and injected subcutaneously into two New Zealand black rabbits. Starting three weeks after the primary injection, the rabbits received booster injections every 2 weeks with similar SoxR preparations in incomplete Freund's adjuvant. Polyclonal antisera were extracted periodically starting 4 weeks after the first injection, and SoxR-specific antibodies were enriched 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 following standard procedures (25).

For immunoblotting, samples of cell suspensions or purified SoxR were electrophoresed in SDS–polyacrylamide gels, and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) with a TE series Transphor electrophoresis unit (Hoefer Scientific, San Francisco, CA). The filters were probed with the affinity-purified antisera and bound antibody was detected with alkaline phosphatase-conjugated anti-rabbit IgG antibodies (Promega, Madison, WI).

EPR spectroscopy

X-band EPR spectra 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 (14,15). The amount of reduced SoxR was determined by comparison in the same experiment to standardized Fe-SoxR samples after reduction with dithionite

(13,14). The high EPR background noise of the cell paste was greatly reduced by electronically subtracting from the spectra for SoxR-containing samples the spectrum of TN5311-pKEN2 cells, which do not express SoxR.

In vitro transcription

The activity of wild-type and mutant SoxR proteins was determined by *in vitro* transcription of the *soxS* gene by commercial *E. coli* RNA polymerase accompanied by the indicated amounts of SoxR, as described previously (13,14).

β-Galactosidase assays

The ability of the mutant proteins to stimulate *soxS* transcription *in vivo* was assessed using TN521 and TN5311. TN5311 cells were transformed either with the pBluescript-based plasmids for low-level SoxR expression, or with the pKEN2-based plasmids for high-level expression. TN521 cells were also transformed with the pSEC119A plasmid. Overnight cultures of the indicated strains were diluted 1:100 (or in some experiments 1:1000) into fresh LB broth containing 50 μ g/ml ampicillin and treated as follows. For TN5311 containing pBluescript-based plasmids, PQ was added to a final concentration of 100 μ M to one aliquot at OD₆₀₀ \pm 0.4, and incubation at 37°C continued 60 min. For TN5311 containing pKEN2-based plasmids, when the cell density approached OD₆₀₀ \pm 0.1, IPTG was added to a final concentration of 0.5 mM and after 60 min PQ was added as above. For TN521 containing pSE380 or pSEC119A, IPTG was added (to 0.5 mM) at OD₆₀₀ \pm 0.05. Aliquots of the cultures were removed at 0, 30 or 60 min following IPTG addition, PQ added (final concentration 250 μ M) and the incubation continued 30 min before harvesting for the assay. β-Galactosidase activity was assayed in SDS/CHCl₃-treated cells as described by Miller (21).

RESULTS

Both strands of the four *soxR* Cys \square Ala mutant genes were sequenced to verify the presence of the mutant codons. Each of the four mutant alleles contained the desired mutations (TGT \square GCT or TGC \square GCC) (Fig. 1) that reprogrammed the cysteine codon to an alanine codon. The full-length sequences of the four mutant constructs showed that no additional mutations were introduced by the site-specific procedure.

In vitro properties of SoxR Cys \square Ala mutant proteins

SoxR proteins for *in vitro* characterization were overexpressed in TN5311 cells (Δ *soxRS*) transformed with pKEN2-derived

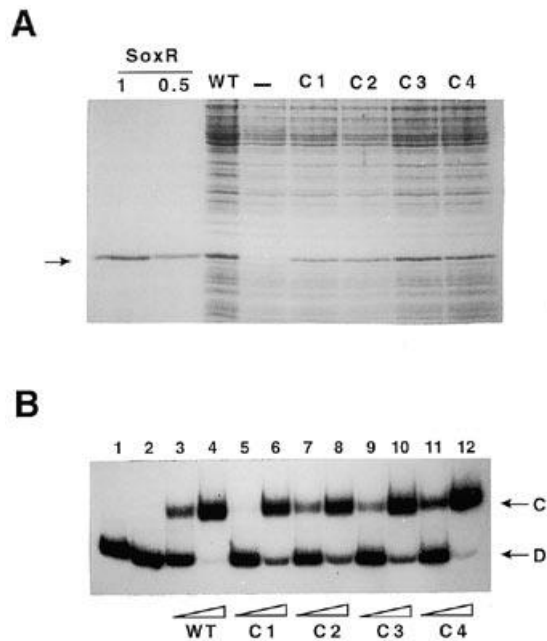


Figure 2. (A) Coomassie blue-stained SDS-polyacrylamide gel of protein extracts from TN5311 cells overexpressing wild-type and mutant SoxR proteins. SoxR, 1 or 0.5 [g purified wild-type SoxR protein. Cell extracts (15 [g): WT, pKOXR plasmid; -, pKEN2 plasmid; C1, pKENC119A plasmid; C2, pKENC122A plasmid; C3, pKENC124A plasmid; C4, pKENC130A plasmid. □, the electrophoretic position of purified SoxR. (B) Binding activity for *soxS* promoter DNA SoxR-containing cell extracts. Lane 1, DNA alone; lane 2, vector control (5 [g); lanes 3 and 4, wild-type SoxR extract (WT); lanes 5 and 6, SoxR-C119A extract (C1); lanes 7 and 8, SoxR-C122A extract (C2); lane 9 or 10, SoxR-C124A extract (C3); lanes 11 and 12, SoxR-C130A extract (C4); odd numbered lanes correspond to 0.5 [g extract protein, even numbered lanes to 5 [g. C, SoxR-DNA complex; D, free DNA.

vectors encoding either wild-type SoxR (pKOXR) or the respective mutant proteins encoded by pKENC119A, pKENC122A, pKENC124A and pKENC130A. SDS-PAGE analysis of cell extracts demonstrated the presence of similar amounts of the wild-type and the four cysteine mutant proteins, each with $M_r = 17\ 000$ (identical to that of wild-type SoxR; Fig. 2A).

We sought to determine whether elimination of the cysteine residues might impair the ability of the mutant proteins to bind *soxS* promoter DNA *in vitro* in electrophoretic mobility shift assays (EMSA) (11). Cell-free extracts containing wild-type and mutant SoxR proteins displayed similar levels of *soxS* binding activity *in vitro* that increased with the amount of extract protein added (Fig. 2B). The mutant and wild-type proteins therefore bind the *soxS* target with similar affinities, since the proteins were present at similar levels in the extracts (Fig. 2A).

Since the Cys□Ala mutant SoxR proteins retained DNA binding activity, all four proteins were extensively purified (to $\geq 90\%$ homogeneity), in parallel with wild-type SoxR and in similar yield (data not shown). The purified mutant proteins each lacked the visible absorbance characteristic of oxidized Fe-SoxR and had no significant Fe detectable by inductively coupled plasma emission spectrometry (< 0.1 atom Fe per SoxR monomer; data not shown). It seemed possible that the Cys□Ala mutant proteins might contain iron *in vivo*, but lose the metal during purification. Analysis by EPR spectroscopy of intact cells overexpressing the various proteins showed that all the Cys□Ala

mutants lacked the near-axial resonance spectrum characteristic of reduced wild-type Fe-SoxR (12–15), while such a spectrum was easily seen in cells overexpressing the wild-type protein (Fig. 3). For the wild-type protein overexpressed *in vivo* in the absence of PQ treatment, 36% was detected as the reduced Fe-SoxR form, consistent with other experiments performed in a similar manner (18).

From the foregoing results, there was no indication that the mutant proteins contained [2Fe-2S] centers *in vivo*, although the unlikely possibility remains that such centers are present but remain in an EPR-silent (oxidized?) state. *In vitro* transcription assays (14) showed that the Cys□Ala mutant proteins were devoid of transcriptional activity for the *soxS* gene, and behaved similarly to apo-SoxR (Fig. 4). As found for the crude extracts (Fig. 2B), binding activity of the purified mutant proteins for the *soxS* promoter was indistinguishable from that of wild-type SoxR (data not shown). Thus, the Cys□Ala mutant proteins neither have tightly bound [2Fe-2S] centers nor function as specific transcription factors for the *soxS* gene *in vitro*.

In vivo properties of the Cys□Ala mutant proteins

In view of the lack of transcriptional activity of the mutated SoxR proteins *in vitro*, we wished to determine whether the Cys□Ala mutant proteins retained ability to induce regulated expression of the *soxS* gene *in vivo*. For this purpose we used a $\Delta soxRS$ strain harboring a single copy of the *soxS2::lacZ* fusion (TN5311), in which expression of β -galactosidase activity encoded by the fusion depends on the *in vivo* activity of SoxR expressed in *trans* (20,26). The *in vivo* effectiveness of the mutant SoxR proteins was initially examined using constructs in the pBluescript vector, which lacks a strong inducible promoter and expresses wild-type SoxR at a level similar to cells containing a single *soxR*⁺ allele in the genome (E.H. and B.D., unpublished data). All four mutant proteins failed to increase β -galactosidase activity above the basal level observed with the vector alone, even when the cells were grown in the presence of PQ, which strongly activated *soxS* expression in cells with wild-type SoxR (< 14 -fold; Fig. 5A).

The results were somewhat different when intracellular SoxR concentrations were elevated (to $< 5\%$ of the total cell protein) using the high level expression vector pKEN2 (11,13) (see Fig. 2A for SoxR protein levels). Wild-type SoxR (expressed from this vector) yielded < 19 -fold *soxS* activation in response to PQ (Fig. 5B), giving an induced level of β -galactosidase expression nearly twice that seen for cells bearing the wild-type pBluescript construct (Fig. 5A). Thus, the normal intracellular concentration of SoxR may be limiting for *soxS* activation in some situations. In contrast, cells overexpressing the mutant proteins from the pKEN2 vector exhibited elevated *soxS* expression that was not affected by PQ (Fig. 5B). For three of the mutant proteins (C119A, C122A and C124A) the level of *soxS2::lacZ* expression was < 4 -fold higher than the basal (uninduced) expression mediated by wild-type SoxR; for the C130A protein, basal expression was < 2 -fold higher (Fig. 5B). The Cys□Ala mutant proteins have therefore lost responsiveness to the redox signal generated by PQ, but can exert a higher basal transcriptional activity than wild-type SoxR.

It seemed possible that expression of the mutant proteins might interfere with wild-type SoxR, either through the formation of heterodimers or by competition for binding the *soxS* promoter. At low levels of mutant SoxR expression (from the pBluescript

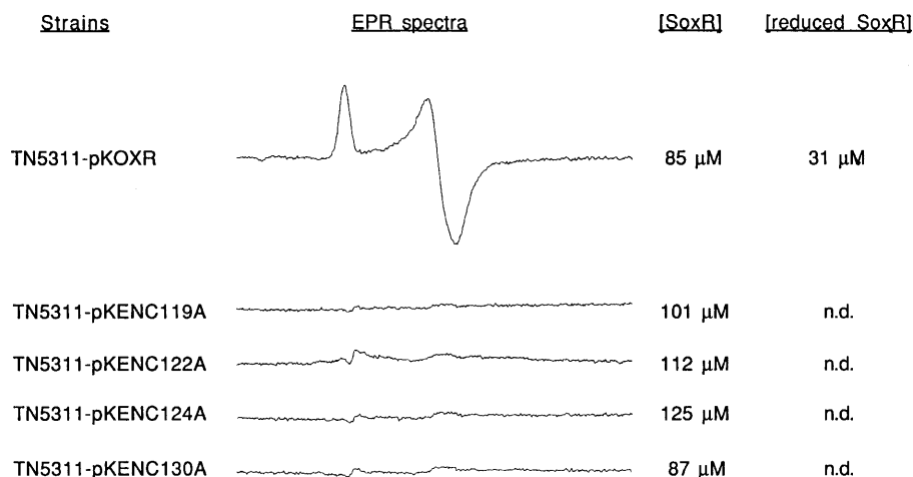


Figure 3. EPR analysis of SoxR *in vivo*. The EPR spectra of cell suspensions were recorded as described previously for purified Fe-SoxR (13). The intracellular SoxR concentration [SoxR] was estimated by immunoblotting of whole cell extracts (see Materials and Methods). The content of reduced [2Fe-2S] SoxR in the cells was estimated from the amplitude of the EPR resonance at g_y (15) normalized to a known preparation of purified SoxR.

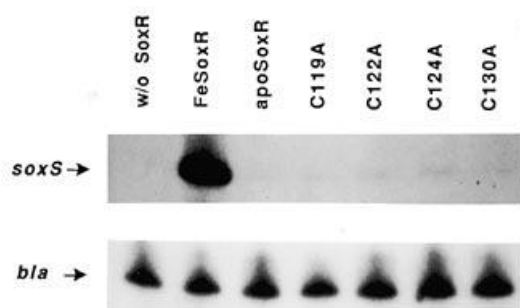


Figure 4. SoxR-dependent *in vitro* transcription of *soxS*. Fe-SoxR (active), apo-SoxR (inactive) or Cys \square Ala mutant proteins (5 ng each) were incubated with 100 nM RNAP in *in vitro* transcription reactions containing a plasmid carrying both the SoxR-dependent *soxS* gene and the SoxR-independent *bla* gene as indicated in Materials and Methods. The corresponding primer extension reactions (13,14) for the *soxS* and *bla* transcripts are indicated.

vectors), induction of *soxS* by wild-type SoxR in response to PQ was unaffected (data not shown).

An additional study was conducted to assess the effect of overexpressing one of the mutant proteins (C119A) on the inducibility of *soxS* in TN521 (*soxR*⁺) cells. Because this strain harbors only the *lacI*⁺ allele, we used the vector pSE380 (bearing *lacI*^q; see Materials and Methods) to achieve tightly regulated SoxR expression inducible by IPTG. After 1000-fold dilution of overnight cultures into fresh medium and outgrowth for 60 min, IPTG was added, followed at various times by a 30 min exposure to PQ (250 μ M) to measure activation of *soxS* transcription. The induction of *soxS2::lacZ* by PQ was essentially the same for both the vector control (pSE380) and the strain overexpressing wild-type SoxR (from pSXR) throughout the course of the experiment (Fig. 6). In contrast, the expression of the C119A protein strongly interfered with *soxS* induction, even at the earliest time point (some leaky expression of SoxR is expected even in the absence of IPTG). PQ inducibility was completely eliminated after only a 30 min pre-induction of the mutant protein with IPTG (Fig. 6). As seen in Figure 5B, basal expression of

β -galactosidase from *soxS2::lacZ* was increased to <1000 U in cells overexpressing the C119A protein.

DISCUSSION

Our previous work established that the SoxR [2Fe-2S] centers are essential for the protein's transcriptional activity at *soxS in vitro* (11,13–15). Here, we have extended the demonstration of this requirement to the activity of SoxR *in vivo* by engineering mutant genes that direct the synthesis of SoxR derivatives with individual cysteine residues replaced by alanines. For at least the C119A derivative, high level synthesis interfered with the normal activity of SoxR during activation by PQ. Despite their inability to be activated by oxidative stress, overexpression of the Cys \square Ala SoxR proteins revealed an unexpectedly high level of basal transcriptional activity *in vivo* that was not detected *in vitro*.

Removal of the SoxR [2Fe-2S] clusters *in vitro* by aerobic exposure of the protein to 2-mercaptoethanol (11,13) or the biological thiol glutathione (15) eliminates the transcriptional activity at *soxS* without any apparent effect on SoxR protein stability, oligomeric state or binding affinity for the *soxS* promoter. Elimination of detectable metal binding by Cys \square Ala replacements effectively blocked SoxR activation in response to PQ and did not significantly affect protein expression *in vivo* or *soxS* binding *in vitro*. These observations provide new evidence for post-translational activation of SoxR, and show that this activation *in vivo* depends critically on the integrity of the [2Fe-2S] centers. The results also support the conclusion from EPR spectroscopy that all four SoxR cysteine residues are involved in anchoring the metal center. Efforts to assemble [2Fe-2S] centers into the Cys119 \square Ala protein *in vitro* were unsuccessful (14).

Re-insertion *in vitro* of [2Fe-2S] centers into wild-type apo-SoxR restored full transcriptional activity (14). However, the properties of the Cys \square Ala mutant proteins alone do not resolve whether the critical step for SoxR activation *in vivo* is synthesis of the [2Fe-2S] centers or a subsequent redox reaction. We have previously suggested (13,14) that these two steps might be linked if the stability of the metal centers is influenced by oxidation and

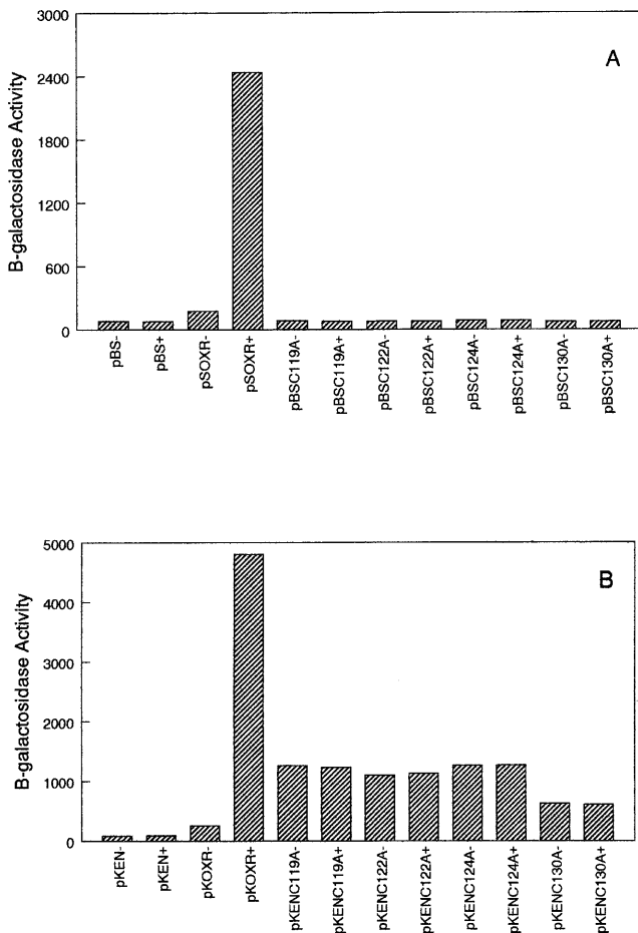


Figure 5. Expression of *soxS2::lacZ* as a function of mutant SoxR expression in strain TN5311 ($\Delta soxR$). (A) Low level expression via uninduced pBluescript vectors or (B) high level expression via induced pKEN2 vectors. +, incubation with PQ; -, non-exposed cells. \odot -galactosidase activity is expressed as Miller units (21).

reduction. However, recent experiments (16,17) indicate that SoxR with reduced [2Fe-2S] centers is stable, but transcriptionally inactive *in vitro*. *In vivo* studies of the properties of constitutive forms of SoxR extend this model to living cells (18).

For wild-type SoxR, the quantity of reduced [2Fe-2S] centers detected in untreated cells corresponded to only 36% of the total SoxR protein (Fig. 3). The EPR-silent forms include both oxidized and apo-SoxR (12,13), with only the former active *in vitro* (11,16,17). The *in vivo* EPR analysis shown here and elsewhere (18) thus supports the conclusion that reduced Fe-SoxR is transcriptionally inactive in cells. Some oxidation evidently occurs during sample preparation by the method employed here: using a modified method (H.D. and B.D., manuscript in preparation), we have measured levels of reduced wild-type protein representing $\geq 95\%$ of the total SoxR.

Two additional features of the SoxR Cys \square Ala mutants merit mention. First, during activation by PQ, none of the substituted forms, when expressed at approximately wild-type levels, competed detectably with wild-type SoxR expressed from a single-copy gene; such interference was observed only upon high level expression of the C119A derivative. In view of their evidently normal stability and DNA binding affinity, the relative

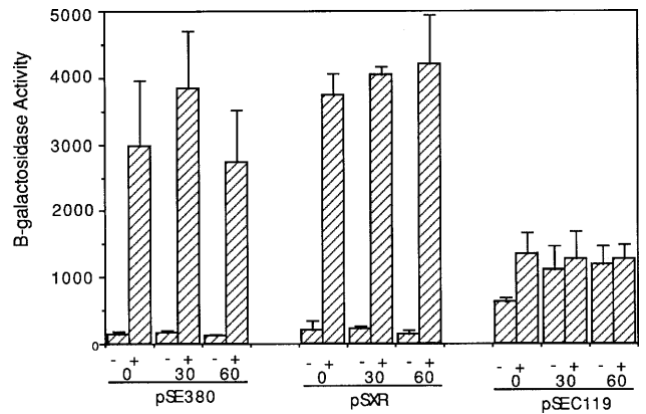


Figure 6. Interference of SoxR-C119A protein with wild-type SoxR *in vivo*. The bottom lines indicate the plasmid present (pSE380, control; pSXR, wild-type SoxR; pSEC119, SoxR-C119A) in strain TN521 (*soxR⁺ soxS2::lacZ*), the time of IPTG treatment to induce recombinant SoxR, and exposure to PQ (+). \odot -Galactosidase activity is shown in units (21). All values represent the mean of three independent samples, except pSEC119/0 min/no PQ, for which only two samples were available.

ineffectiveness of the mutant proteins in blocking *soxS* activation by wild-type SoxR is striking. In principle, the mutant proteins could interfere with wild-type SoxR in two ways: by the formation of mixed dimers with a wild-type subunit, or by competition for binding the *soxS* promoter. It would thus be of interest to determine whether the mixed dimers retain partial or complete function. Regarding the second possible mode of interference, wild-type SoxR and the Cys \square Ala mutant proteins bound the *soxS* promoter equally well *in vitro* (Fig. 2B). However, RNAP binds the *soxS* promoter cooperatively with activated SoxR (11), an effect that could disfavor competition by low levels of the Cys \square Ala mutant proteins.

The second unexpected feature of the Cys \square Ala mutant forms of SoxR was the relatively high basal *soxS* transcription they exert upon overexpression *in vivo*, which contrasts with the essentially undetectable activity of these proteins *in vitro*. It remains possible that the Cys \square Ala mutant proteins cause transcriptional activation higher than that of apo-SoxR, but at a level still undetectable in our *in vitro* assay. Alternatively, the *in vivo* activity of the mutant proteins in the absence of a redox signal could suggest the existence of additional controls on *soxS* activation that are overcome by high concentrations of the Cys \square Ala derivatives but not non-activated wild-type SoxR. The known negative autoregulation by SoxS (27) is evidently not responsible for this effect, which we observed in SoxS deficient cells. Previous genetic studies (2,3) identified regulatory mutations only in *soxR* and *soxS* (9,10), but a smaller effect on *soxS* expression caused by other mutations might have been missed. One possible negative regulator is Rob protein, which is abundant [< 5000 molecules/cell; (28)] and has DNA binding specificity that overlaps that of SoxS (29-31). MarA protein also exhibits overlapping specificity with SoxS (32), but its expression is kept low through repression by the MarR protein (33).

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