SoxR protein, a member of the MerR family of transcriptional activators, mediates a global oxidative stress response in *Escherichia coli*. Upon oxidation or nitrosylation of its [2Fe-2S] centers SoxR activates its target gene, *soxS*, by mediating a structural transition in the promoter DNA that stimulates initiation by RNA polymerase. We explored the molecular basis of this signal transduction by analyzing mutant SoxR proteins defective in responding to oxidative stress signals *in vivo*. We have confirmed that the DNA binding domain of SoxR is highly conserved compared with other MerR family proteins and functions in a similar manner to activate transcription. Several mutations in the dimerization domain of SoxR disrupted intersubunit communication, and the resulting proteins were unable to propagate redox signals to the *soxS* promoter. Mutations scattered throughout the polypeptide yielded proteins that were under-responsive to *in vivo* redox signals, which indicates that the redox properties of the [2Fe-2S] centers are influenced by global protein structure. These findings indicate that SoxR functions as a redox-responsive molecular switch in which subunit interactions transduce a subtle alteration in oxidation state into a profound change in DNA structure.

For single-celled organisms, genetic responses to environmental change are exquisitely fine-tuned because the environment can change quickly and dramatically. This holds true for responses to oxidative stress, which arise when the balance between the production and destruction of unstable oxygen derivatives is upset (1). Reactive oxygen species are generated during normal aerobic metabolism (2) by physical agents such as UV radiation (3) or by environmental compounds such as paraquat (PQ)\(^1\) and phenazine methosulfate (PMS). The latter compounds divert electrons to oxygen to generate superoxide, thereby depleting cellular pools of NAD(P)H in a process called redox-cycling (4).

When cellular oxidative stress increases, SoxR, a dimeric protein that functions as a redox-responsive genetic switch, mediates transcriptional regulation of the *soxRS* regulon that encodes resistance to oxidative stress in *Escherichia coli* and *Salmonella enterica* (5). The SoxR protein remains tightly bound to its target *soxS* promoter in the absence and presence of oxidative stress (6, 7). Each subunit of SoxR contains a redox-active [2Fe-2S] center that is anchored by four cysteine residues located in the C terminus of the 154-residue polypeptide (8). Oxidation of these centers or direct nitrosylation by nitric oxide activates SoxR to stimulate transcription of the *soxS* gene, the product of which activates downstream members of the regulon (for review, see Ref. 9).

The MerR family proteins, to which SoxR belongs, regulate genes that confer protection against various environmental stresses. MerR (10), ZntR (11), and CueR (12) bind metal ions to activate their respective metal resistance systems, whereas BmrR (13) and MtaN (14) bind organic compounds and activate multidrug resistance genes in *Bacillus subtilis*. Promoters regulated by members of the MerR family contain an unusual 19-bp separation of the −35 and −10 promoter elements, which is 17 bp in most ε\(^−\)-RNA polymerase (RNAP)-regulated promoters. The 19-bp spacer prevents open complex formation by RNAP in the absence of an activator (15). MerR family members bind to a sequence of dyad symmetry located between the −35 and −10 promoter elements of their target promoters and upon activation, in response to the appropriate stimulus, distort and remodel their target promoters to make them better substrates for RNAP and dramatically increase the transcription initiation rate. Evidence for these conformational changes is provided by biochemical studies of SoxR, MerR, and ZntR (11, 16, 17). Further evidence for the DNA-distortion mechanism of transcription activation is provided by the crystal structures of BmrR and MtaN, solved in complex with their target promoters, which show local DNA unwinding and base pair disruption, resulting in the realignment of the −35 and −10 sites (18, 19).

MerR family proteins bear clear sequence homology in the ~110-residue N-terminal domain. This segment contains a winged helix-turn-helix DNA binding motif and the dimerization region, which comprises half of an antiparallel coiled-coil in the crystal structures of BmrR, MtaN, CueR, and ZntR (18–21). The variable length C-terminal domains of MerR-family proteins contain coactivator binding elements and share little sequence similarity.

With the advent of efficient genome sequencing, the known MerR family of transcriptional regulators is rapidly growing (22). Although the majority of proteins in this family respond to various environmental stimuli, SoxR is the only identified member that responds to oxidative stress. SoxR can be further sub-categorized with MerR family proteins that activate transcription in response to metal ions (including MerR, ZntR, and CueR). An important distinction between these proteins and SoxR, however, is that whereas the other proteins are activated upon binding the appropriate metal, the SoxR [2Fe-2S] centers are present even in the inactive state (23). It is the chemical modification (one-electron oxidation/reduction or nitrosylation) of these centers that regulates SoxR transcriptional activity (6, 24, 25).
To better understand the mechanism of gene activation by MerR family members and, in particular, signal transduction via protein iron-sulfur centers, we previously isolated 29 soxR mutant alleles that confer defects in activating soxS expression in response to oxidative or nitrosative stress in vivo (7). The resulting mutant proteins might suffer from one or a combination of the following defects; inability to make appropriate contacts with the soxS promoter, inability to remodel the promoter and signal RNAP to initiate transcription, loss or destabilization of [2Fe-2S] centers, intact [2Fe-2S] centers that are poorly responsive to redox/nitric oxide signals, inappropriate regulation by cellular reducing systems, and inability to undergo conformational changes to the activated form. In this work we have assessed the specific defects in 19 mutant alleles using a variety of in vitro and in vivo biochemical assays. Viewed in a structural context provided by MerR family proteins, these results underscore the critical effect of global protein conformation on the redox reactivity of the SoxR [2Fe-2S] centers and the role of subunit interactions in the transmission of oxidative stress signals.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of soxR Genes—For molecular analysis, the wild-type (WT) soxR gene, 19 soxR mutant alleles defective in activating soxS transcription in response to PQ-induced stress (7), and the C124A mutant gene (8) were subcloned into the pET16b using a variety of work we have assessed the specific defects in 19 mutant alleles using a variety of in vitro and in vivo biochemical assays. Viewed in a structural context provided by MerR family proteins, these results underscore the critical effect of global protein conformation on the redox reactivity of the SoxR [2Fe-2S] centers and the role of subunit interactions in the transmission of oxidative stress signals.

Whole Cell Electron Paramagnetic Resonance (EPR) Spectroscopy—E. coli BL21(DE3) cells expressing His-tagged SoxR proteins from plT16b were used for in vivo EPR experiments. 100-ml cultures of the expression strain or control strain with the empty plT16b vector were grown in LB medium described above. Cells were harvested by centrifugation (10 min at 1000 x g), and resuspended in 50 mM NaH2PO4, pH 8.0, 300 mM NaCl at one-hundredth the original volume. To achieve oxidation of the [2Fe-2S] centers, cultures were treated with 15 μM FAD for 20 min with aerobic before sampling. Concentrated cells (0.25–0.4 ml) were placed in EPR tubes (Wilmad) and flash-frozen in liquid nitrogen.

EPR measurements were made at Massachusetts Institute of Technology’s Department of Chemistry Instrumentation Facility on a Bruker Model ESP-300 equipped with a flow cryostat. EPR measurement conditions were as follows; microwave frequency, 9.38 GHz; microwave power, 6.3 milliwatts; modulation frequency, 100 kHz; modulation amplitude, 1.2 millitesla; sample temperature, 30 K; receiver gain, 2 x 104.

SoxR concentration in the cells was determined by immunoblot analysis with an anti-SoxR antibody (Amerham Biosciences) and development with Amerham Biosciences ECF reagent. Previously quantified His-tagged SoxR was used as a standard. The EPR spectrum for untreated cells containing empty vector was subtracted from the spectra of cells expressing SoxR proteins and were normalized for protein concentration.

In Vitro Transcription and Primer Extension—In vitro transcription reactions were performed using a previously described procedure (16, 28), except that the RNAP complexes were challenged with 50 μg/ml heparin sulfate before the addition of the four ribonucleotide triphosphates. The RNAP (Epicenter) was then added (final concentration of 68 nm), and the reactions were incubated for 15 min at 37 °C. RNAP (10 μM) was preincubated with 50 μg/ml heparin sulfate before the addition of 1 μl of freshly prepared 50 μM KNO3 and incubation for 3 min at room temperature. Reactions were terminated by the addition of 2 μl of β-mercaptoethanol and 6 μl of 0.5 % EDTA and extracted with phenol-chloroform. The DNA (200 μl) was precipitated by the addition of 1 ml of a mixture containing 95% ethanol, 0.15 M sodium acetate, pH 5.2, and 5 μg/ml RNase A and incubation at −80 °C. The pellets were washed with 70% ethanol, dried under vacuum, and resuspended in 100 μl of 1 M piperidine. After a 30-min incubation at 90 °C, the piperidine was removed by lyophilization, the reaction was washed with 100 μl of water, and the DNA was precipitated and washed with ethanol as before. Finally, the pellet was resuspended in 10 μl of loading buffer (98% formamide, 10 mM EDTA, 0.025% xylene cyanole FF, 0.025% bromphenol blue), loaded on a 10% polyacrylamide sequencing gel containing 8 M urea, 120 mM Tris borate, and 1.2 mM EDTA, pH 8, and electrophoresed at 65 watts constant power. The same DNA was also cleaved in Maxam-Gilbert guanine- and guanine + adenine-specific reactions for use as a DNA sequence ladder (28). After drying, the gel was applied to an Amersham Biosciences PhosphoImager, and the signals were digitized and analyzed using ImageQuant.

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with purified His-tagged SoxR protein and a 32P-labeled 180- bp fragment of the soxS promoter as previously described (27).

KMoO4 Footprinting—KMnO4-footprinting reactions were carried out with the 180-bp fragment soxS promoter that was used in the electrophoretic mobility shift assay. The template and non-template strands were 32P-labeled and PCR amplified as previously described (27), and the PCR products were purified using a kit from Qiagen.

Footprinting reactions were carried out in 20 μl of buffer (10 mM Tris, pH 7.5, 75 mM KCl, 2.5 mM MgCl2, 1 mM dithiothreitol, and 1 mM DMAP as a DNase I inhibitor). Labeled DNA (2 nm) and His-tagged SoxR proteins were added to the buffer and incubated for 10 min at room temperature. RNAP (Epicenter) was then added (final concentration of 68 nm), and the reactions were incubated for 15 min at 37 °C. RNAP (10 μM) was preincubated with 50 μg/ml heparin sulfate before the addition of 1 μl of freshly prepared 50 μM KNO3 and incubation for 3 min at room temperature. Reactions were terminated by the addition of 2 μl of β-mercaptoethanol and 6 μl of 0.5 % EDTA and extracted with phenol-chloroform. The DNA (200 μl) was precipitated by the addition of 1 ml of a mixture containing 95% ethanol, 0.15 M sodium acetate, pH 5.2, and 5 μg/ml RNase A and incubation at −80 °C. The pellets were washed with 70% ethanol, dried under vacuum, and resuspended in 100 μl of 1 M piperidine. After a 30-min incubation at 90 °C, the piperidine was removed by lyophilization, the reaction was washed with 100 μl of water, and the DNA was precipitated and washed with ethanol as before. Finally, the pellet was resuspended in 10 μl of loading buffer (98% formamide, 10 mM EDTA, 0.025% xylene cyanole FF, 0.025% bromphenol blue), loaded on a 10% polyacrylamide sequencing gel containing 8 M urea, 120 mM Tris borate, and 1.2 mM EDTA, pH 8, and electrophoresed at 65 watts constant power. The same DNA was also cleaved in Maxam-Gilbert guanine- and guanine + adenine-specific reactions for use as a DNA sequence ladder (28). After drying, the gel was applied to an Amersham Biosciences PhosphoImager, and the signals were digitized and analyzed using ImageQuant.

Electrophoresis on 8% polyacrylamide, 6M urea gels and quantified on a phosphorimager. Previously developed 180-bp fragment

Electrophoretic Mobility Shift Assay—Amersham Biosciences ECF reagent. Previously Quantified His-tagged SoxR was used as a standard. The EPR spectrum for untreated cells containing empty vector was subtracted from the spectra of cells expressing SoxR proteins and were normalized for protein concentration.

In Vitro Transcription and Primer Extension—In vitro transcription reactions were performed using a previously described procedure (16, 28), except that the RNAP complexes were challenged with 50 μg/ml heparin sulfate before the addition of the four ribonucleotide triphosphates. The sox and bla transcripts were quantified by transcription analysis as previously described (16, 28), except that the bla transcript was reverse-transcribed with primer pBH4 (5′-GGGAAATAGCGGACACGGAGCAGGAGTA and X (5′-CATATGCGGACACTCGGAGGCGGACGATCAATCTGCTCCTCCTCGGCAACTTGCGGAGG)3′). The PCR products were amplified and sequenced through the 5′ end of strain EH4 (DeltaoxR derivative of GC4468 containing lysogenized λ (soxS promoter-lacZ reporter construct) (15)) by the method described by Datsenko and Wanner (29). Successful transformants were resistant to 20 μg/ml chloramphenicol, and integration into the targeted rscX site
was verified by colony PCR with primers Y (5'-CATTCCCGTGGAA-CACCATGC-3') and Z (5'-CAACCTTCTGCTGGCGGC-3'), which yielded a 1-kilobase PCR fragment in the correct clones. One such clone, designated EH46rsxC, was used for further analysis.

**β-Galactosidase Analysis**—β-Galactosidase activity in EH46 or EH46rsxC cells expressing the various SoxR proteins from pSE380-based plasmids was determined as previously described (7). Oxidative stress was generated by treatment with 100 μM PQ or 15 μM PMS for 1 h with shaking at 220 rpm.

**Alignment and Structure Modeling**—An alignment of the amino acid sequences of six MerR family members was performed with ClustalW (30) and confirmed by visual inspection. The SoxR polypeptide was modeled on the crystal structure of CueR (21) using SWISS-MODEL and Swiss-PdbViewer (31-33).

### RESULTS

**SoxR Proteins with Defective DNA Binding Ability**—SoxR activates soxS transcription during oxidative stress by stimulating open complex formation at the soxS promoter and increasing the rate of transcription initiation by RNAAP. A mutation in SoxR that compromises its promoter binding ability would obviously cause a defect in transcription-activating ability. In previous work (7) we observed that the in vivo promoter binding ability of many of the SoxR mutant proteins (assessed by their ability to repress lacZ expression from a soxR promoter fusion) was similar to that of WT SoxR protein. Band-shift assays performed with these purified proteins, Q64R, I66T, L84R, L86S, and L94F, E115G, D117G, and R127L, also showed binding constants that were within 4-fold of the WT SoxR value, ranging from 0.8 to 3 nM, indicating that the individual functional domains of SoxR are highly interdependent and that the global conformation of each domain impacts the properties of the others.

**SoxR Proteins Defective in Promoting Open Complex Formation**—KMnO₄ oxidizes mainly unpaired thymines, which are characteristic of initiated, “open” complexes formed by RNAAP. Footprinting reactions with KMnO₄ were performed with [³²P]labeled DNA fragments that contained the overlapping soxS and soxR promoters (Fig. 1A). SoxR binds to a site located in the −10/−35 spacer of the soxS promoter, downstream of the −10 element of the soxR promoter, and exerts nearly constant repression of soxR (34). This repression was also apparent from footprint experiments carried out here. Incubation with RNAAP alone before KMnO₄ exposure resulted in strong thymine reactivities in the −1 and −10 regions of the soxR promoter, indicating open complex formation (Fig. 1B). Of particular note were the reactions at thymine −2, −3, −10, −11, and −13 (bottom strand) and thymine +2, +1, −5, and −8 (top strand).

Pre-incubation of the DNA with SoxR eliminated this footprint, indicating inhibition of transcription initiation by SoxR (Fig. 1B).

Activated SoxR allosterically modifies the suboptimal soxS promoter to make it a better substrate for RNAAP. KMnO₄ footprinting with RNAAP in the absence of SoxR showed no hypersensitive thymine in the soxS promoter on the transcribed or untranslated strands (Fig. 1C; data not shown). Preincubation with purified SoxR, however, resulted in strong reactions at thymine +1, −2, −3, −10, and −12 on the untranscribed strand (Fig. 1C). No footprint was observed on the transcribed strand (data not shown). As expected, DNA binding mutants Y31H, L36V, I62N, A63V, and I73F did not detectably promote open complex formation at the soxS promoter (Fig. 1C), although DNA binding variants G15D, I106T, and C124Y were able to do so when present at higher concentrations (Fig. 1C). Even so, the footprints obtained with the G15D, I106T, and C124Y proteins were weaker than that seen with WT SoxR. Mutant proteins Q64R, I66T, H84R, L86S, L94F, S96P, E115G, D117G, and R127L produced footprints similar to that seen with WT SoxR, which indicates that these proteins can promote open complex formation (Fig. 1C). Mutant proteins L94P and S95P did not produce a footprint characteristic of open complex formation at the soxS promoter (Fig. 1C). Although these two proteins showed somewhat impaired DNA binding activity in vitro (Table I), they displayed normal DNA binding activity in vivo (7), and their inability to activate soxS transcription is unlikely to be due to a DNA binding defect.

**Detection of SoxR [²Fe-²S] Centers in Vivo**—The [²Fe-²S] centers of SoxR are essential cofactors for transcriptional activity; apoSoxR is unable to activate transcription from the soxS promoter in vivo (16). If some of the mutations described here result in a loss of the [²Fe-²S] centers, then those proteins would be unable to activate transcription. The reduced [²Fe-²S] centers of SoxR produce an EPR signal with g values of 1.91, 1.93, and 2.02 (16, 35). We utilized this characteristic to detect [²Fe-²S] centers in the mutant proteins in vivo by performing EPR spectroscopy on intact cells. The presence of strong EPR signals under aerobic, non-stress conditions in WT SoxR and many of the mutant proteins indicated intact [²Fe-²S] centers (Fig. 2). As expected, the C124Y variant that lacks one of the iron ligands did not contain [²Fe-²S] centers, as shown by the absence of a significant EPR signal in cells expressing this protein (Fig. 2). In addition, mutant proteins Y31H, L36V, I62N, A63V, I73F, and I106T showed weak EPR signals in cells expressing them, indicating that these proteins contain only low levels of [²Fe-²S] centers in vivo (Fig. 2 and data not shown).
The [2Fe-2S] centers in purified WT SoxR protein produce a visible absorption spectrum with four maxima at 332, 414, 462, and 548 nm (35, 36). Absorption spectra of the purified mutant proteins (data not shown) corroborated the results obtained from in vivo EPR spectroscopy (Fig. 2 and data not shown).

**Redox Activity of SoxR [2Fe-2S] Centers**—The ability of SoxR to activate transcription is controlled by the oxidation state of its [2Fe-2S] centers, which undergo one-electron oxidation/reduction with a midpoint redox potential of ~285 mV (6, 24, 37, 38). Therefore, the mere presence of [2Fe-2S] centers is not sufficient for SoxR activity, but rather, their ability to undergo oxidation/reduction is a critical feature.

SoxR mutant proteins that contain [2Fe-2S] centers would display a transcription defect if they were under-responsive to redox signals. Such proteins might be predicted to have higher transcriptional activity *in vitro* when exposed to oxygen than they do in a reducing intracellular environment. In comparing the *in vitro* transcriptional activities of the mutant proteins to their activities *in vivo* after activation with 100 μM PQ, we observed that Q64R, H84R, L86S, L94F, S96P, E115G, D117G, and R127L all showed significantly higher transcription *in vitro* (Fig. 3). Variant R127L showed the largest difference (14-fold higher activity *in vitro* than *in vivo*), whereas H84R showed the smallest (1.6-fold). Mutant proteins Q64R, L86S, L94F, S96P, E115G, and D117G; 100 nM, G15D, Y31H, L36V, I62N, A63V, I73F, L94P, S95P, I106T, C124Y, and C124A. Lanes G are guanine-specific sequence ladders; lanes A + G are adenine + guanine-specific sequence ladders.

To determine whether the [2Fe-2S] centers of the SoxR mutant proteins that contain them are redox-active *in vivo*, we again performed EPR analysis. As described above, the reduced [2Fe-2S] centers of SoxR produce a signature EPR spectrum. Oxidation of the [2Fe-2S] centers, however, eliminates this signal (6, 24, 37, 38). Treatment of cells expressing WT SoxR with the potent redox-cycling agent PMS caused a significant decrease in the EPR signal amplitude, indicating oxidation of the [2Fe-2S] centers (Fig. 2). Mutant proteins Q64R, H84R, L86S, L94F, S96P, E115G, D117G, and R127L also appeared to be redox-sensitive, since PMS treatment of cells expressing these proteins caused a substantial decrease in the EPR signal (Fig. 2 and data not shown). The [2Fe-2S] centers of mutant proteins G15D, I66T, L94P, and S95P, which showed similar transcriptional activities *in vivo* and *in vitro*, were also oxidized by PMS treatment (data not shown).

The EPR experiment described above did not eliminate the possibility that the transcription defect in some of the mutant proteins was due to [2Fe-2S] centers that are under-responsive to redox signals *in vivo*. PMS has a midpoint redox potential (+80 mV) significantly higher than that of PQ (−285 mV) (39) or SoxR (−285 mV) (6, 24), and PMS is more effective than PQ at redox-cycling and causing oxidative stress. We used PMS in the *in vivo* EPR experiments, because PQ is not an effective
SoxR-activating agent in *E. coli* B strains such as BL21 (37). We, therefore, assayed β-galactosidase activity in cells expressing WT or mutant SoxR proteins from pSE380-based plasmids after a 1-h treatment with 15 μM PMS. Many of the mutant proteins that had shown a severe translational defect in response to PQ displayed significantly higher activity with PMS, some almost equaling WT activity (Fig. 3). These proteins included Q64R, I66T, H84R, L86S, L94F, S96P, E115G, D117G, and R127L, all of which (except I66T) also showed high in vitro transciptional activity (Fig. 3). Thus, these proteins most likely contain [2Fe-2S] centers with shifted redox potentials and require more oxidizing power than WT SoxR to become activated.

**Effect of Cellular Reducing Systems for SoxR**—An aspect of SoxR regulation that is often overlooked is how the protein is maintained in an inactive state in the absence of oxidative stress. A complex of genes involved in partially maintaining the SoxR [2Fe-2S] centers in the reduced state was recently identified and comprises six members of the *rsc* operon and the *rscC* gene product (40). Deletion of any one of these genes increased the basal activity of SoxR by 20% (40).

The transcription defect of some of the SoxR mutant proteins could be due to their preferential reduction in *vivo*, which would be overcome by exposure to air during purification. Proteins potentially in this category (higher transcriptional activity in *vitro* than in *vivo*) would include Q64R, H84R, L86S, L94F, S96P, E115G, D117G, and R127L (Fig. 3). To test this possibility, we constructed a strain in which the *rscC*-coding region was replaced with the *cat* gene in strain EH46 (see “Experimental Procedures”). The resulting mutant strain and the parent EH46 strain were transformed with pSE380-based vectors containing the WT *soxR* gene or the mutant alleles encoding the proteins mentioned above, and the transcription-activating abilities of the various proteins in the two strains was determined by assaying β-galactosidase activity from the *soxS* promoter-lacZ reporter. As previously observed (40), WT SoxR showed significantly higher activity in an *rscC* background, which indicates loss of regulation (Fig. 4). Of the mutant proteins only H84R and D117G displayed a similar deregulation in the *rscC* background, although neither protein generated β-galactosidase activity comparable with that of WT SoxR (Fig. 4). The other SoxR mutant proteins analyzed showed similar transcriptional activity in the WT and *rscC* backgrounds and in every case much lower than that observed for WT SoxR (Fig. 4).

**DISCUSSION**

The SoxR protein functions as a transcriptional activator under conditions of oxidative stress. This protein has so far resisted detailed structural characterization, but we have used a combination of approaches to define residues that are important to the specific SoxR functions of transcriptional activation, dimerization, DNA binding, and iron binding. Fig. 5A shows an alignment of the SoxR polypeptide with five other MerR family members, CueR, ZntR, MerR, MtaN, and BmrR. The crystal structures of CueR and ZntR (21) and of BmrR and MtaN (complexed with their target promoters) (18, 19) yielded the secondary structural elements shown. SoxR and MerR are predicted to adopt a similar fold, and this is apparent in a structural model of SoxR that was created using CueR structural coordinates as template (Fig. 5, B–E). In the CueR structure, the metal binding domain (MBD) of one monomer was well ordered, whereas the corresponding residues in the other monomer were disordered (21). For this reason we were unable to model the MBD and more C-terminal residues in one of the subunits of SoxR (Fig. 5, B–E).

The putative DNA binding domain (DBD) of SoxR consists of residues 14–81 predicted to fold into the winged helix-turn-helix motif peculiar to MerR family members (Fig. 5B). Residues in this region of BmrR and MtaN (marked by # in Fig. 5A) make specific contacts with DNA in the crystal structures (18, 19). Many mutations in this region of SoxR yielded DNA binding-defective proteins (Y31H, L36V, I62N, A63V, and I73F) that were, therefore, unable to stimulate transcription initiation by RNAP (Table I; Fig. 1C). Most of these residues (except Ile-62) are functionally conserved among MerR family proteins (Fig. 5A). The DNA binding defect in the G15D protein was
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Three independent experiments. Below, a fold difference in activities between the two backgrounds is indicated.

The assays contained 68 nM RNAP and 250 nM SoxR protein. The amount of soxS mRNA is reported as a percent of the amount obtained with 250 nM His-tagged WT SoxR protein. The results represent the means and S.D. (bars; some are not visible on this scale) of three independent experiments.

FIG. 3. Transcriptional activity of SoxR mutant proteins in vitro and in vivo with PQ and PMS. β-Galactosidase activities in PQ-treated cells were previously determined (7). Briefly, EH46 cells expressing the soxR alleles from pSE380-based plasmids were treated with 100 μM PQ for 1 h before the assay for β-galactosidase activity (open columns), which are reported as a percent of the activity obtained with WT SoxR (6500 Miller units = 100%). β-Galactosidase activity in SoxR-expressing EH46 cells treated with 15 μM PMS for 1 h are depicted by gray columns and are reported as a percent of the activity obtained with WT SoxR (7000 Miller units = 100%). In vitro transcriptional activities of His-tagged SoxR proteins (black columns) were determined as described under “Experimental Procedures.” The assays contained 68 nM RNAP and 250 nM SoxR protein. The amount of soxS mRNA is reported as a percent of the amount obtained with 250 nM His-tagged WT SoxR protein. The results represent the means and S.D. (bars; some are not visible on this scale) of three independent experiments.

FIG. 4. Transcriptional activities of SoxR proteins in WT and rrsxC mutant cells. β-Galactosidase activities from a soxS promoter-lacZ fusion were determined in strains EH46 (open columns) and EH46rrsxC (black columns) expressing WT and mutant SoxR proteins expressed from pSE380-based plasmids. Cells were grown under normal aerobic conditions in the absence of additional oxidative stress. The -fold difference in activities between the two backgrounds is indicated below the graph. The results shown represent the mean and S.E. of three independent experiments.

Partial activity of SoxR proteins expressed at high concentrations, where it weakly promoted open complex formation by RNAP (Fig. 1C). The low level of [2Fe-2S] centers in some of the DNA binding-defective proteins was unexpected (Fig. 2). In CucR, the MBD is stabilized by interactions with the DBD (21), and a similar situation may exist in SoxR where the MBD and C-terminal residues of one monomer appear to pack against the DBD of the other subunit (Fig. 5C). Disruption of these interactions may explain the destabilization of the [2Fe-2S] centers in the Y31H, L36V, I66T, and I106T proteins.

Two mutations in the putative DBD of SoxR that did not cause a DNA binding defect were Q64R and I66T (Table I). In the activated BmrR and MtnR structures, the DNA adopts a distorted conformation that is stabilized by interactions between the phosphate backbone and specific residues in the DBD including Lys-60 in BmrR (Lys-56 in MtnR) (18, 19). Residue Gln-64 of SoxR corresponds to Lys-60 of BmrR (Fig. 5A), and changing this residue to arginine would maintain a hydrogen bond in this position that can stabilize the distorted DNA in the open complex (Fig. 1C). The Q64R protein displayed properties similar to WT SoxR in vitro yet showed a very weak response to PQ in vivo (Fig. 3). The [2Fe-2S] centers of Q64R may be hyposensitive to redox signals because the transcription defect in vivo was abrogated upon exposure to air or in cells treated with PMS (Fig. 3). PMS, as a more effective redox-cycling agent than PQ, would generate higher oxidative stress intracellularly and, thus, activate SoxR more efficiently. Measurement of the midpoint potential of Q64R will show whether such hyposensitivity can be correlated with a redox shift (38). The I66T variant showed similar properties to Q64R, except that the I66T protein had a severe transcription defect in vitro (Fig. 3). Although this protein, like Q64R, may respond inefficiently to redox signals, its low in vitro transcriptional activity suggests that the I66T protein may also have some structural instability that is exacerbated by handling.

The remaining mutations are in regions outside the putative DBD of SoxR. Residues 87–119 are predicted to form a helix in the open complex (Fig. 1C). The Q64R protein displayed properties similar to WT SoxR in vitro yet showed a very weak response to PQ in vivo (Fig. 3). The [2Fe-2S] centers of Q64R may be hyposensitive to redox signals because the transcription defect in vivo was abrogated upon exposure to air or in cells treated with PMS (Fig. 3). PMS, as a more effective redox-cycling agent than PQ, would generate higher oxidative stress intracellularly and, thus, activate SoxR more efficiently. Measurement of the midpoint potential of Q64R will show whether such hyposensitivity can be correlated with a redox shift (38). The I66T variant showed similar properties to Q64R, except that the I66T protein had a severe transcription defect in vitro (Fig. 3). Although this protein, like Q64R, may respond inefficiently to redox signals, its low in vitro transcriptional activity suggests that the I66T protein may also have some structural instability that is exacerbated by handling.
Figure 5. Functional consequences of substitutions in the SoxR polypeptide. A, sequence alignment of MerR family members SoxR, CueR, ZntR, MtbR, MtaN, and BmrR. The first 100 residues of the 279-residue BmrR polypeptide are shown. Secondary structure elements from crystallographic analyses of BmrR (18), MtaN (19), ZntR, and CueR (21) are indicated below the sequences by arrows for \( \beta \) strands and boxes for \( \alpha \) helices; MBD denotes the metal binding domain in the metal-activated proteins. Functionally similar residues are in boldface; hyphens indicate gaps; asterisks depict SoxR mutations that were analyzed in this work; pound signs mark BmrR residues that contact DNA. B-D, a structural model of SoxR was created using SWISS-MODEL and SWISS-Pdb Viewer with CueR coordinates as template (21). The DBD, dimerization (Dimer) and MBD domains are indicated. Side chains (wild-type) are included only for the residues changed by mutation. E, mutations that resulted in proteins that showed higher transcription-activating ability in vitro than in vivo with PQ. The redox properties of these proteins have most likely been altered with respect to WT SoxR. F, mutations that resulted in proteins that were unable to transmit oxidative stress signals to the soxS promoter to activate gene transcription. See "Discussion" for details.
that were unable to promote transcription under all conditions tested. The proline in these proteins may introduce conformational rigidity that prevents the transition to the active form of the protein-DNA complex. If Leu-94 is mutated to phenylalanine instead, the resulting protein is able to activate ssoS transcription under certain conditions (see below). Similarly, replacing Ser-95 with leucine instead of proline changes the redox potential of the resulting protein, making it more easily oxidized than WT SoxR, and the S95L protein displays constitutive activity under conditions where WT SoxR is inactive (38). The I106T, L194P, and S95P mutations underscore the importance of interactions between the two subunits of SoxR that allow propagation of the redox signal to the ssoS promoter (Fig. 5E).

Other changes in the putative dimerization domain of SoxR (L94F, S96P, E115G, D117G), in the linker connecting the DBD with the dimerization domain (H84R, L86S), and the R127L mutation in the MBD, displayed a phenotype similar to that of Q64R, a severe transcription defect in response to PQ that was significantly abrogated by replacing Ser-95 with leucine instead of proline changes the redox potential of the resulting protein, making it more easily oxidized than WT SoxR, and the S95L protein displays constitutive activity and not merely by local geometry (Fig. 5D). This suggestion is not without precedence since the S95L mutation mentioned above resulted in a hyperactive protein due to an altered molecular machine in which subunit interactions tune redox activity and transform a subtle change in oxidation state into a profound effect on DNA structure.

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In this work we also explored the effect of a recently identified reducing system on SoxR (40). The products of the rsa operon and the rscE gene are partially responsible for maintaining SoxR in a reduced state in the absence of oxidative stress (40), although no direct interactions have been identified between any of the gene products and SoxR. We considered the possibility that the in vivo transcription defect displayed by certain SoxR mutant proteins might be caused by excessive reduction of these proteins by the rsa/rscE system. A mutation in the rsaC gene caused an increase in the basal activity of WT SoxR and in mutant proteins H84R and D117G (Fig. 4). The activity of the two mutant proteins, however, was significantly lower than that of WT SoxR, and the deficiency in these proteins, therefore, cannot be ascribed solely to a defect in their regulation by the rsa/rscE complex.

This work identifies residues in the SoxR polypeptide that are critical for transducing signals of oxidative stress into transcriptional activation of a target gene. Our analysis shows that redox signals are transmitted to the DNA via the subunit dimerization domain; mutations that disrupt communication between the two subunits of SoxR result in proteins that are unable to transmit such signals. Furthermore, the redox properties of the [2Fe-2S] centers are apparently influenced by residues in all three functional domains of SoxR. Thus, the SoxR homodimer is a compact molecular machine in which subunit interactions tune redox activity and transform a subtle change in oxidation state into a profound effect on DNA structure.

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