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A cluster of constitutive mutations affecting the C-terminus of the Redox-sensitive SoxR transcriptional activator

Tatsuo Nunoshiba and Bruce Demple*
Department of Molecular and Cellular Toxicology, Harvard School of Public Health, Boston, MA 02115, USA

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ABSTRACT

Activation of *Escherichia coli* oxidative stress regulon genes (*sodA*, *zwf*, *fumC*, *nfo*, etc.) is mediated by a two-stage regulatory system: the redox-sensitive SoxR protein transcriptionally activates the *soxS* gene, whose product then stimulates transcription of the regulon genes. Previous experiments showed that limited 3′ truncation of *soxR* gene causes constitutive *soxRS* expression. DNA sequence analysis of the *soxR* genes from the *soxRS*-constitutive strains isolated originally (Greenberg *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87, 6181–6185) revealed that three alleles encode amino acid substitutions or a chain termination clustered near the C-terminus of SoxR. Two other single-amino-acid substitutions in constitutive alleles mapped to the helix-turn-helix motif and to a region of unknown function in the center of the polypeptide, respectively. No constitutive mutation was found within the region encoding the cysteines of the SoxR FeS center, in the *soxR* or *soxS* promoters, or in the *soxS* structural gene. Since an in-frame deletion of just nine SoxR residues (136–144; full-length SoxR = 154 residues) gave rise to a powerful constitutive allele, it appears that a small segment of the SoxR C-terminus maintains the protein in the inactive state. Conversely, an intact C-terminus is evidently not required for gene activation by SoxR.

INTRODUCTION

Exposure of *Escherichia coli* to redox-cycling agents such as paraquat (PQ), which strongly elevate the intracellular flux of superoxide (1), specifically induces the synthesis of ~40 proteins (2,3). Among these, a group of nine proteins is controlled by the two-stage *soxRS* system (4–7). This coregulated group of gene products includes manganese-containing superoxide dismutase, the oxidative DNA repair enzyme endonuclease IV, glucose-6-phosphate dehydrogenase (G6PD), and fumarase C (8). In this system, the SoxR protein acts as a redox-sensitive transcriptional activator of the *soxS* gene (9,10). The induced SoxS protein transcriptionally activates the various *soxRS* regulon genes (11,12) and negatively regulates its own transcription (13).

SoxR protein has homology with the MerR protein family (11). Binding of Hg^{2+} by MerR causes the protein to activate transcription of the *mer* operon, which provides for detoxification of this noxious metal (14). The SoxR–MerR homology includes a predicted helix-turn-helix motif that probably mediates specific DNA binding, and some C-terminal cysteine residues that allow metal binding by these proteins. In the case of SoxR, this metal is iron, and SoxR contains a redox-active iron–sulfur (FeS) cluster (15). Although removal of the FeS center does not detectably diminish SoxR affinity to the *soxS* promoter, only oxidized Fe-SoxR stimulates transcription initiation at *soxS in vitro* (up to 100-fold) (15). Redox modulation of the FeS center may thus be employed to modulate the SoxR activity *in vivo* but this remains to be established (15).

In previous studies (6,11), it was noticed that short deletions into the 3′ end of the *soxR* gene, which led to the attachment of vector-derived oligopeptides, resulted in mutant proteins with strong constitutive activity ($SoxR^c$). These limited observations left open at least two possibilities: either the loss of SoxR sequences or the attachment of new sequences to the SoxR C-terminus might have caused the constitutive activity. We addressed this question by constructing new mutant alleles that encode SoxR proteins with modified C-termini and analyzed their *in vivo* activity. We also determined the nature of the mutations in a set of $SoxR^c$ strains isolated by phenotypic selection (4). Three of these 'naturally' isolated alleles (of five total) affected the same small segment implicated by deletion analysis to be involved in SoxR activation.

MATERIALS AND METHODS

Strains and plasmids

The *E.coli* strains used in this study are listed in Table 1.

In addition to the previously described plasmids pCA2710, pCA2711 (11) and pTN2712 (13), pTN2713 was newly constructed by removing the *Sma*I–*Kpn*I fragment from pCA2710, end-blunting with T4 DNA polymerase, and religation. The deletion in plasmid pTN2713 specifies an in-frame removal of nine amino acids from SoxR (see Results). Two

*To whom correspondence should be addressed

Table 1. The *Escherichia coli* strains used in this study

Strains	Relevant genotypes	References
GC4468	*soxRS*$^{+}$	(4)
DJ901	as GC4468 but Δ(*soxRS-zjc2204*) *zjc2205*::Tn*10*Km	(4)
JTG1048	as GC4468 but *soxR104 zjc2206*::Tn*10*Km	(4)
JTG1050	as GC4468 but *soxR102 zjc2206*::Tn*10*Km	(4)
JTG1052	as GC4468 but *soxR101 zjc2206*::Tn*10*Km	(4)
JTG1069	as GC4468 but *soxR103 zjc2206*::Tn*10*Km	(4)
JTG1078	as GC4468 but *soxR105 zjc2206*::Tn*10*Km	(4)
QC1709	as GC4468 but λΦ(*sodA'*::*lacZ*)	(16)
TN530	as GC4468 but λΦ(Δ*soxR soxS'*::*lacZ*)	(13)
TN531	as DJ901 but λΦ(Δ*soxR soxS'*::*lacZ*)	(13)
TN1530	as TN530 but *soxR101 zjc2206*::Tn*10*Km	This study
TN1799	as QC1709 but Δ(*soxRS-zjc2204*) *zjc2205*::Tn*10*Km	This study
TN2530	as TN530 but *soxR102 zjc2206*::Tn*10*Km	This study
TN3530	as TN530 but *soxR103 zjc2206*::Tn*10*Km	This study
TN4530	as TN530 but *soxR104 zjc2206*::Tn*10*Km	This study
TN5315	as TN531 but *recA56 srlC300*::Tn*10*	This study
TN5530	as TN530 but *soxR105 zjc2206*::Tn*10*Km	This study

synthetic oligonucleotides containing a few mismatches to generate *Eco*RI and *Hind*III sites (primers c and d in ref.11) were used as primers in polymerase chain reactions (PCR) (17). Using these primers, DNA containing whole coding region of *soxR* gene along with the *soxS* promoter was amplified by Taq polymerase using the genomic DNA of the SoxR^{c} mutants as a template. The PCR fragments were purified with a PCR Purification Kit (QIAGEN Inc., Chatsworth, CA), digested with *Eco*RI and *Hind*III, and cloned into *Eco*RI−*Hind*III digested plasmid vector pSE380 (Invitrogen, San Diego, CA), which contains both the *lacI*-regulated *trc*-promoter and the *lacI*^{q} gene (18). After transformation into strain TN5315, individual ampicillin-resistant colonies were screened on MacConkey plates for elevated basal expression of β-galactosidase from the resident *soxS'*::*lacZ* fusion (13). These plasmids were designated pTN101−pTN105 and, after purification using a QIAGEN Mid-prep Kit, were sequenced using Sequenase Kit Version 2 (United States Biochemical, Cleveland, OH).

Toxicity measurements

The sensitivity of various strains to the redox-cycling agent phenazine methosulfate (PMS) and the antibiotic nalidixic acid (Nal) was examined by measuring bacterial growth on gradient plates prepared as described previously (11). The gradient plates were supplemented with 100 μg/ml ampicillin, 50 μg/ml kanamycin and/or 50 μg/ml tetracycline as appropriate.

Cell extracts and enzyme assays

Overnight cultures in LB medium (18) supplemented with the appropriate antibiotics were diluted 100-fold into 30 ml fresh medium and grown at 37°C to reach OD_{600} ~0.5. The preparation of cell extracts and measurement of protein concentrations were carried out as previously described (11). G6PD activity was monitored by following the production of NADPH at 340 nm (19). Fumarase C activity was assayed in cell extracts after storage overnight at 4°C followed by incubation at 37°C for 1 hr, a treatment that inactivates the fumarase AB activity (8). The fumarase activity was determined by following the production of L-fumarate from L-malate at 250 nm (20). β-Galactosidase activity in cells permeabilized with sodium dodecyl sulfate-$CHCl_3$ was assayed as described previously (9,18).

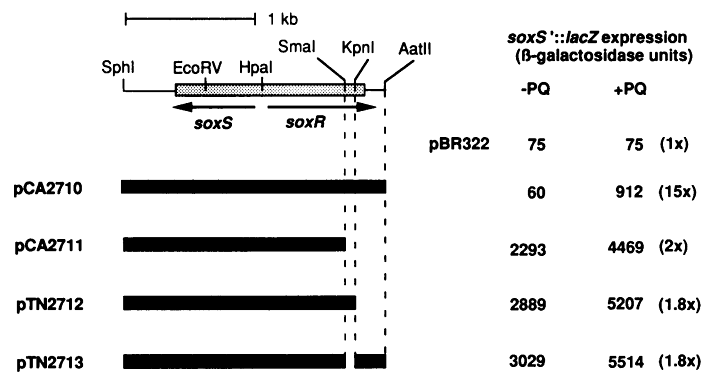

Figure 1. Effect of multicopy *soxR* plasmids on expression of a *soxS'*::*lacZ* fusion. TN531 [Δ*soxRS* λΦ(Δ*soxR soxS'*::*lacZ*)] bearing the indicated plasmids (all in the vector pBR322) were treated with 50 μM PQ for 60 min. The values represent the averages of determinations from at least three independent experiments, which agreed within 11%. The numbers in parentheses are the fold induction in PQ-treated cells. The results for plasmids pCA2710, pCA2711 and pTN2712 were as reported in reference 13.

RESULTS

Modification of the SoxR C-terminus

Limited deletion/replacement mutations engineered into the 3′ end of the *soxR* gene caused constitutive expression of the *soxRS* regulon (6,11,13). This effect was mediated by the high-level expression of the *soxS* gene, dependent on the altered *soxR* gene (9,10). These deletions apparently convert SoxR protein to the activated form in the absence of the redox signal required for the wild-type SoxR protein (9). However, it was not established whether these constitutive alterations result from the loss of the C-terminus of SoxR or from the addition of an oligopeptide tail encoded by the vector.

To clarify this point, we examined two other modified plasmids (pTN2712 and pTN2713) with limited 3′ deletions in *soxR* (see Fig. 1). These plasmids and the parental plasmid pCA2710 and the vector (pBR322), were introduced into TN531 [Δ*soxRS* λΦ(Δ*soxR soxS'*::*lacZ*)] and TN1799 [Δ*soxRS* λΦ(*sodA'*::*lacZ*)]. As expected, the basal expression of *soxS'*::*lacZ* was similar in the strains with the vector alone or the *soxR*$^{+}$ plasmid

Table 2. Constitutive phenotype of the strains bearing mutant SoxR plasmids

Plasmids	Phenotypic resistance[a]		G6PD (Units/mg)	Fumarase C (Units/mg)	*sodA'*::*lacZ* expression[b]
	PMS	Nal			
pBR322	36	21	0.23 (1.0)	5.0 (1.0)	254(1)
pCA2710	65	37	0.28 (1.2)	14.3 (2.1)	666(3)
pCA2711	88	68	0.95 (4.1)	48.7 (9.7)	4068(16)
pTN2712	87	70	1.03 (4.5)	47.6 (9.5)	4081(16)
pTN2713	90	72	1.16 (5.0)	49.5 (9.9)	4155(16)

Each value is an average obtained from two independent experiments. Except for *sodA'*::*lacZ* expression, all measurements were performed with transformants of strain TN531. The ratios in parentheses were calculated by dividing each value by that of the parent strain with pBR322. The strains were grown without PQ.

[a]Resistances to PMS or Nal are given as the growth of these strains on gradient plates (see methods), expressed as a percentage of the gradient.

[b]For *sodA'*::*lacZ* expression, the plasmids were introduced into strain TN1799 [Δ*soxRS* λΦ(*sodA'*::*lacZ*)].

pCA2710, but was induced 15-fold by PQ treatment only in the latter strain (Fig. 1). No induction of *soxS* by PQ was observed in the strain with only the vector. In contrast, the modified *soxR* plasmids, pCA2711, pTN2712 and pTN2713, caused high-level *soxS'*::*lacZ* expression even in the absence of PQ and still higher after treatment (Fig. 1). These latter strains also showed various other constitutive phenotypes: elevated resistance to PMS or Nal, high activities of G6PD and fumarase C, and a high basal expression of the *sodA* gene (Table 2).

The SoxR proteins encoded by pCA2711 and pTN2712 are predicted to have eight- and fifteen-amino-acid oligopeptide tails replacing, respectively, 19 and 11 C-terminal residues of the wild-type polypeptide (Fig. 2). Since the *KpnI–SmaI* deletion causes an in-frame deletion of just nine residues (Fig. 2), the elimination of a short segment rather than the addition of a new peptide evidently renders the constitutive activity of SoxR.

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           115              130              145
wild type  E LDGCI GCGCL SRSDC PLRNP GDRLG EEGTG ARLLE DEQN*
pCA2711    E LDGCI GCGCL SRSDC PLRNP ILEDE RAS*
pTN2712    E LDGCI GCGCL SRSDC PLRNP GDRLG EEGFL KTKGP RDTPI FIG*
pTN2713    E LDGCI GCGCL SRSDC PLRNP ----- ----G ARLLE DEQN*
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Figure 2. Predicted amino acid sequences in the C-terminus of modified SoxR proteins. The cluster of cysteine residues in $SoxR^+$ is in bold. Italicized letters represent residues encoded by the vector DNA. The asterisks mark the polypeptide C-termini, and the dashes correspond to deleted residues.

Mutations in *soxRS*-constitutive strains

A set of $SoxR^c$ strains was originally isolated as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced or spontaneous mutants resistant to menadione, a superoxide-generating agent (4). These mutants have elevated resistance to other oxidative agents (PMS, plumbagin, *t*-butyl hydroperoxide) and to multiple antibiotics, accompanied by high levels of oxidative defense enzymes such as G6PD, Mn-containing superoxide dismutase and the DNA repair enzyme endonuclease IV (4,5). Five independent mutations, *soxR101–soxR105*, were mapped to the *soxRS* locus (4).

At least two possible targets for *soxRS*-constitutive mutations need to be considered: the *soxS* promoter region and the *soxR* structural gene. Promoter mutations in *soxS* could elevate basal transcription; mutations in *soxR* could trigger its transcription-activating function in the absence of redox signals. In order to distinguish these possibilities, the mutations were transduced into strains TN530 [$soxRS^+$ λΦ(Δ*soxR soxS'*::*lacZ*)] and QC1709 [$soxRS^+$ λΦ(*sodA'*::*lacZ*)]. The presence of the mutations was verified by phenotypic resistance to Nal and by elevated G6PD and fumarase C activities (Table 3). Promoter mutations in *soxS* would be expected to activate only the *sodA* fusion via increased production of SoxS protein, which would not activate the *soxS* fusion. Mutations that activate SoxR would increase expression of both the *sodA* and the *soxS* fusion. All five mutations caused a high basal expression of both *sodA'*::*lacZ* and *soxS'*::*lacZ* (Fig. 3). The order of potency of the constitutive mutations was *soxR102* ≅ *soxR105* > *soxR101* ≅ *soxR103* > *soxR104* for all

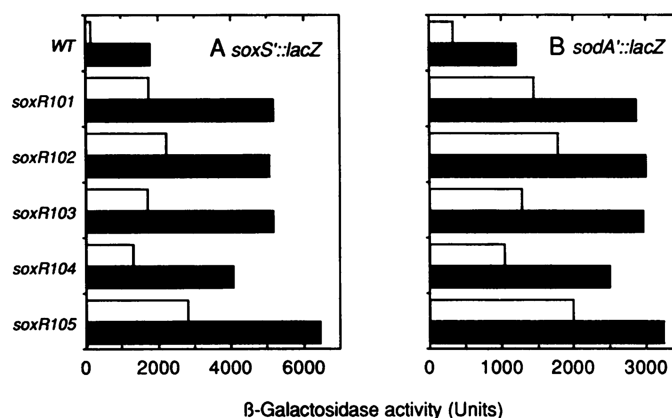

Figure 3. Effect of chromosomal *soxR* alleles on *soxS'*::*lacZ* and *sodA'*::*lacZ* fusions. Strains TN530 [$soxRS^+$ λΦ(Δ*soxR soxS'*::*lacZ*)] and QC1709 [$soxRS^+$ λΦ(*sodA'*::*lacZ*)] were transduced with the indicated *soxRS* alleles and examined for β-galactosidase activity, with or without a prior PQ treatment (50 μM for 60 min). The values represent the averages of determinations from at least three independent experiments, which agreed within 8% for *soxS* and 14% for *sodA*. Open bars, not treated; hatched bars, PQ-treated.

phenotypes examined (Fig. 3 and Table 3). Further induction of both *lac* fusions could be achieved by treatment of the cells with PQ (Fig. 3). Thus, all five mutations are probably in the *soxR* gene and cause activation of SoxR in the absence of inducers, which then acts *in trans* on the *soxS'*::*lacZ* reporter gene.

Cloning of *soxR*^{c} mutations and DNA sequence analysis

We were unable to obtain stable transformants bearing multicopy plasmids with the whole *soxRS* region from the constitutive

Table 3. Phenotypes of TN530 bearing the *soxR*[c] mutants *in cis*

Strains	Nal resistance[a]	G6PD (Units/mg)	Fumarase C (Units/mg)
TN530 (wild type)	30	0.25 (1.0)	6.3 (1.0)
TN1530 (*soxR101*)	68	0.48 (1.9)	36.8 (5.8)
TN2530 (*soxR102*)	78	0.59 (2.4)	54.0 (8.6)
TN3530 (*soxR103*)	72	0.45 (1.8)	34.0 (5.4)
TN4530 (*soxR104*)	54	0.40 (1.6)	19.3 (3.1)
TN5530 (*soxR105*)	86	0.54 (2.2)	57.2 (9.1)

Each value is an average obtained from two independent experiments. The ratios in parentheses were calculated by dividing each value by that obtained for strain TN530.
[a]Resistance to Nal is given as the extent of growth on Nal gradient plates (see methods), expressed as a percentage of the gradient.

Table 4. Effect of SoxR[c] mutations on *soxS'*::*lacZ* expression

Plasmids	*soxS'*::*lacZ* −PQ	+PQ	Type of mutation amino acid change
pSE380	75	75	
pSXR	200	2627	wild type
pTN101	3370	6657	GGT⇒GAT [^{143}Gly⇒sp]
pTN102	4467	6890	CGC⇒TGC [^{20}Arg⇒Cys]
pTN103	2915	6284	GGT⇒GAT [^{143}Gly⇒Asp]
pTN104	2085	5222	TCG⇒TTG [^{95}Ser⇒Leu]
pTN105	4119	6629	TTA⇒TAA [^{139}Leu⇒Stop]
pTN102R-7	314	464	CGC⇒TGC [^{20}Arg⇒Cys] GGC⇒GAC [^{123}Gly⇒Asp]

Each value is an average obtained from two independent experiments. The strain TN5315 [*recA* Δ*soxRS* λΦ(Δ*soxR soxS'*::*lacZ*)] bearing the indicated plasmids was treated with or without 50 μM PQ for 60 min, and β-galactosidase activity measured.

strains, perhaps due to toxic effects of SoxS overproduction (11). We therefore cloned the PCR-amplified *soxR* gene and the 5′ end of *soxS* into plasmid pSE380 under control of the IPTG-inducible *trc* promoter, accompanied by the *lacI*[q1] allele (17). The resulting plasmids were placed into a Δ*soxRS recA* strain bearing λΦ(Δ*soxR soxS'*::*lacZ*) (TN5315). Even in the absence of IPTG, the expression of *soxS'*::*lacZ* in TN5315 bearing these plasmids was 10- to 20-fold higher than that conferred by plasmid pSXR (11), a pSE380 derivative carrying the wild type *soxR* gene (Table 4). The same order of potency described above for the constitutive mutations in the chromosome was observed in these expression plasmids (Table 4). As seen for the chromosomally-located mutations, the plasmid-borne *soxR* alleles could also be further activated by PQ treatment (Table 4). Increasing the level of *soxR* in these strains by incubation with IPTG further elevated *soxS'*::*lacZ* expression only ~1.5-fold in the absence of PQ, and ~2-fold for PQ-treated cells (21).

DNA sequence analysis of the mutant alleles revealed individual mutations only in the *soxR*-coding region (Fig. 4), with none found in the *soxS* promoter. Only one point mutation was observed in each *soxR*[c] allele. It was striking that, of the five mutations, three (*soxR101*, *soxR103* and *soxR105*) were found in the same nine-codon region at the 3′ end of *soxR* whose deletion causes a constitutive phenotype (Fig. 2; Fig. 4). Although isolated independently (4), *soxR101* and *soxR103* had the same mutation causing a substitution of glycine-143 by aspartic acid (Fig. 4). The *soxR105* allele is predicted to produce a truncated protein of 138 residues by virtue of an *ochre* mutation within the same small region. The *soxR102* mutation converted

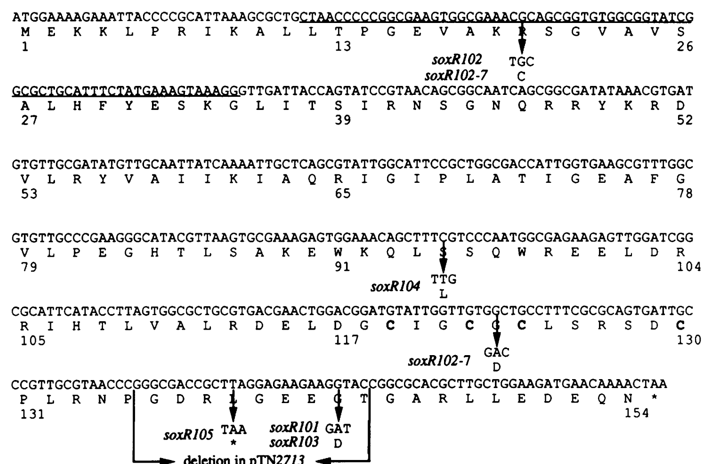

Figure 4. Constitutive mutations in the *soxR* gene. The underlining indicates the predicted helix-turn-helix motif (6,11). The mutation (*soxR102-7*) that reverses the constitutive activity of *soxR102* and the deletion in pTN2713 are also shown. Asterisks denote stop codons. The entire *soxR* gene and the *soxS* promoter were sequenced on both strands of each clone. SoxR residue numbers are shown below the amino acid sequence.

arginine-20 to cysteine in the helix-turn-helix motif, and *soxR104* changed serine-95 to leucine.

During the screening process while cloning *soxR*-constitutive alleles, we also obtained a derivative of *soxR102*, *soxR102-7*, which had lost most of its effect on the basal expression of *soxS'*::*lacZ* (reduced to ~300 units of β-galactosidase activity;

Table 4). When a strain with pTN102-7 was treated with PQ, only a 1.5-fold induction of *soxS'::lacZ* was observed (Table 4). The *soxR102-7* allele contains both the original mutation of *soxR102* and a second point mutation in the center of the cysteine cluster (glycine-123 to aspartic acid; Fig. 4).

DISCUSSION

We have characterized the nature of eight mutations in the *soxR* gene that allow SoxR protein to act as a transcriptional activator in the absence of redox-cycling agents. The most striking observation is that six of these mutations involve the same 3'-terminal region of the *soxR* gene (Fig. 4). Since a nine-codon segment of *soxR* can be deleted to yield the constitutive activity (as in pTN2713), the corresponding peptide is evidently not required for DNA binding or transcriptional activation by SoxR. In fact, to judge from the constitutive behavior of the pC-A2711-encoded protein, the C-terminal 19 amino acids (one eighth of the SoxR polypeptide) are not required for activating the *soxS* promoter. Each of the constitutive mutant proteins retained a limited ability to undergo additional activation in response to paraquat.

What might be the function of C-terminal region of SoxR? SoxR binds between the −35 and −10 positions of the *soxS* promoter and activates transcription by stimulating initiation (rather than DNA binding) by RNA polymerase (15). SoxR protein contains iron-sulfur (FeS) centers essential for this activating process (15). The transcriptionally active form of SoxR has oxidized FeS centers (15), and recent data indicate that the non-activated form of the protein *in vivo* contains reduced FeS centers (22). Consequently, SoxR might be activated by oxidation of its FeS centers by superoxide.

In this context, several possibilities exist to explain the constitutive activity of the mutant proteins characterized here. For example, the C-terminal region might function to maintain non-activated SoxR in the inactive conformation. In this model, oxidation of the FeS centers would switch SoxR to the activated form, and this requirement would be alleviated by mutations in the C-terminal region and perhaps other sites. However, preliminary experiments have undermined this idea by revealing that the constitutive activities of *soxR101−soxR105* all depend on aerobic growth (21).

More unusual mechanisms can be envisioned related to the redox biochemistry of SoxR. Wild-type SoxR is not significantly activated by normal aerobic growth, but one could envision mutant proteins hypersensitive to oxidation. Such proteins might then be activated even during normal aerobic growth. Such hypersensitivity might result from heightened reactivity with low levels of the 'normal' activators (perhaps superoxide (9) or nitric oxide (23)), or from reactions with molecules that normally do not activate, such as O_2 (9). The loss or alteration of polypeptide structure near the cysteine cluster of SoxR (Fig. 4) might cause such altered reactivity.

Alternatively, there is a strong possibility that SoxR is maintained in the inactive state by a reductase that counteracts spontaneous oxidation (15). Alterations of SoxR's C-terminus might then diminish the effectiveness of such a reductase, perhaps by disrupting interaction between the two proteins. Again, changes in the polypeptide near the cysteine cluster are obvious candidates to affect such an interaction, but similar effects could also be exerted by the point mutations in *soxR102* and *soxR104*. It should be noted that independent searches for *soxR*-constitutive mutants yielded only mutations at the *soxRS* locus (4,5). Reductase-deficient mutants might have been expected if there were one major, nonessential activity specific for SoxR. Studies now underway should help clarify whether such a reductase pathway exists.

The powerful activating effect of individual *soxR*-constitutive mutations is striking in comparison to experience with the homologous MerR protein. Single amino-acid substitutions in MerR gave only weak constitutive activity (24) and had to be combined to generate a strong effect (25). This difference might derive from the repressor activity that characterizes non-activated MerR, but which is absent from SoxR (9). A second difference relates to the activation mechanisms of these two proteins. MerR is activated by metal binding, while SoxR is apparently activated by a redox reaction involving an existing metal center (15,22). Perhaps the binding energy of Hg^{2+} for MerR exerts a significant conformational effect that is already potentiated by the presence of FeS centers in the SoxR protein, and which is unleashed by oxidation of the iron−sulfur centers.

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