Thiol-Mediated Disassembly and Reassembly of [2Fe-2S] Clusters in the Redox-Regulated Transcription Factor SoxR†

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ABSTRACT: SoxR, a transcription factor containing [2Fe-2S] clusters, governs the cellular response to oxidative stress in *Escherichia coli*. The oxidation state of the iron−sulfur clusters regulates the SoxR transcriptional activity. When the reduced iron−sulfur clusters become oxidized ($[2Fe\text{-}2S]^{2+}$ state), SoxR is activated to stimulate transcription of the *soxS* gene, whose product in turn switches on a group of genes encoding various proteins that defend against oxidative stress and antibiotics. A previous study showed that the oxidized [2Fe-2S] clusters of SoxR are destroyed by a free-radical-dependent process in vitro during aerobic exposure to the biological thiol glutathione. Here, we show that different thiols have differing effects on the SoxR [2Fe-2S] clusters. Like reduced glutathione, *N*-acetyl-L-cysteine, L-cysteine methyl ester, and L-cysteine ethyl ester disrupted the SoxR [2Fe-2S] clusters in aerobic solution. This disruption was blocked by L-cysteine, which was effective at concentrations 100-fold lower (1−10 μM) than the disrupting thiols (1 mM). In view of a previous observation that superoxide dismutase and catalase block the disruption process, this result suggests that L-cysteine may quench reactive SoxR or thiol intermediates involved in the cluster disruption reaction, the detailed mechanism of which remains unknown. In contrast, bifunctional thiols such as dithiothreitol or dithioerythritol promoted the aerobic assembly of the functional [2Fe-2S] clusters into apo-SoxR in the presence of Fe^{2+} and inorganic sulfide. The dithiol protein thioredoxin-A of *E. coli* acted catalytically in vitro in the presence of thioredoxin reductase and NADPH to promote [2Fe-2S] cluster assembly into apo-SoxR. The regulatory activity of SoxR in vivo, assessed by monitoring the paraquat-mediated induction of a *soxS′::lacZ* reporter fusion, was significantly lower in a strain lacking both thioredoxin-A and glutathione reductase, which maintains reduced glutaredoxins. Thus, cellular monothiols and dithiol proteins may contribute to SoxR regulation by affecting the disassembly and reassembly of the [2Fe-2S] clusters.

The primary cellular response to oxidative stress in *Escherichia coli* involves the rapid transcriptional activation of defense genes (*1*). Oxidative stress-responsive gene expression has also been observed in eukaryotic cells, but the signal transduction processes have not been fully established (*2*, *3*). A well-understood example in *E. coli* is the redox-sensitive transcription activator SoxR,[1] a putative sensor of intracellular superoxide stress (*4*, *5*). When cells are exposed to agents such as paraquat (PQ), which redox-cycles to generate a powerful superoxide flux intracellularly (*6*), SoxR becomes activated and stimulates the transcription of *soxS*, its only known target gene (*7*, *8*). The *soxS* gene product activates at least twelve genes, whose products metabolize excess superoxide, repair oxidatively damaged DNA, and replenish NADPH and Krebs cycle activities. The antioxidant functions increase cellular resistance to oxidants such as PQ and to nitric oxide-generating macrophages (*9*). Activation of this defense system also increases resistance to various nonoxidant antibiotics (*1*).

SoxR protein is a homodimer, and each monomer (M_r ∼17000) contains one redox-active [2Fe-2S] cluster (*10*, *11*) with a redox midpoint potential of −285 mV (*12*, *13*). The four cysteine residues in the SoxR polypeptide provide the ligands for the [2Fe-2S] clusters, as shown by a site-directed mutagenesis study (*14*). The [2Fe-2S] clusters are essential for transcriptional activity of SoxR in vitro (*10*, *15*) and in vivo (*14*). When the [2Fe-2S] clusters are chemically oxidized under anaerobic conditions, SoxR becomes reversibly activated to stimulate the transcription of its target gene *soxS* (*12*, *13*). In vivo EPR measurement of the SoxR [2Fe-2S] clusters demonstrated that the SoxR [2Fe-2S] clusters are predominantly in the reduced state during normal aerobic growth (*4*, *5*, *16*) and become rapidly oxidized (activated) when cells are exposed to PQ or other redox-cycling agents (*16*). These results show that the [2Fe-2S] clusters are the redox switch in the SoxR activation process.

Unlike the [4Fe-4S] cluster in the anaerobic regulatory factor Fnr, which is very sensitive to oxygen (*17*), SoxR [2Fe-2S] clusters are stable in aerobic solution. Moreover,

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[1] Abbreviations: EPR, electron paramagnetic resonance; Fnr, fumarate and nitrate reductase regulator; GSH, glutathione; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid; PQ, paraquat; SoxR, superoxide-regulated transcription factor in *E. coli*; SoxS, SoxR-regulated transcription factor in *E. coli*.

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H_2O_2 (up to 500 μM) or superoxide (generated by hypoxanthine and xanthine oxidase) in solution had little effect on the oxidized [2Fe-2S] clusters of SoxR (*15*). Surprisingly, the SoxR [2Fe-2S] clusters were disrupted when the protein was incubated with the thiol compounds 2-mercaptoethanol (*18*) or reduced glutathione (GSH) (*15*). This disruption process of the SoxR [2Fe-2S] clusters required oxygen and could be inhibited by catalase or superoxide dismutase (*15*). Furthermore, addition of H_2O_2 or a superoxide-generating system to mixtures of SoxR and GSH greatly accelerated the disruption reaction. These features suggested that the [2Fe-2S] clusters are not extracted from SoxR by thiols as described for ferredoxin (*19*), but rather that thiol-based free radicals are responsible for the disruption of the SoxR [2Fe-2S] clusters. Although the complex chemistry of thiol-mediated disruption of the SoxR [2Fe-2S] clusters is not established, one possibility is that the reaction proceeds through a redox-mediated ligand exchange (*15*).

Here, we extend these studies to other thiols, which reveals dramatic differences in their effects on the SoxR [2Fe-2S] clusters. Three classes of thiol activity are shown: monothiols that, like GSH, disrupt the SoxR [2Fe-2S] clusters; L-cysteine, which plays a protective role against the first class; and dithiols that mediate reassembly of functional [2Fe-2S] clusters into apo-SoxR.

MATERIALS AND METHODS

SoxR Purification. SoxR protein was over-produced and purified as described previously (*10*, *15*). Apo-SoxR (SoxR without [2Fe-2S] clusters) was prepared as follows: SoxR (10 μM) dissolved in a buffer containing 500 mM NaCl and 50 mM Hepes-NaOH (pH 7.6) was treated with 1 mM reduced GSH at 37 °C for 45 min and diluted with an equal volume of 50 mM Hepes-NaOH (pH 7.6). The mixture was loaded on a 5 mL column of P-11 phosphocellulose (Whatman International Ltd., England) equilibrated with 100 mM NaCl, 50 mM Hepes-NaOH (pH 7.6) and washed with 200 mL of the same buffer before eluting with 500 mM NaCl, 50 mM Hepes-NaOH (pH 7.6). The purity of SoxR in these experiments (typical final concentration of 10 μM in reaction) was >95% as judged by Coomassie staining of SDS−polyacrylamide gels.

Spectroscopy. A UV−vis spectrophotometer (Perkin-Elmer Lambda 3A) was used to measure SoxR visible absorbance spectra and to follow the kinetics of SoxR [2Fe-2S] cluster assembly and disruption as described previously (*15*). The X-band electron paramagnetic resonance (EPR) spectra of the SoxR [2Fe-2S] clusters were obtained using a Bruker model ESP-300 equipped with an Oxford Instruments 910 continuous flow cryostat (courtesy of Professor J. Stubbe's laboratory, Chemistry Department, Massachusetts Institute of Technology). For EPR measurement, SoxR samples were reduced with 1 mM freshly prepared sodium dithionite before transfer to EPR tubes and freezing. Routine EPR measurement conditions were as follows: microwave frequency, 9.47 GHz; microwave power, 1 mW; modulation frequency, 100 kHz; modulation amplitude, 1.2 mT; sweep field, 310−370 mT; sample temperature, 20 K; and receiver gain, 10^5.

Reaction of Thiols with SoxR. Typically, a final concentration of 1 mM thiol from a freshly prepared stock solution (100 mM) was added to 1 mL of purified SoxR (10−20 μM) in a buffer containing 500 mM NaCl and 50 mM Hepes-NaOH (pH 7.6). The reactions were either incubated in 37 °C for 30 min, or transferred to an open-top cuvette for UV−vis spectroscopy (at room temperature) to monitor the absorbance change of the oxidized SoxR [2Fe-2S] clusters. Reduced thioredoxin was prepared by mixing 800 μM thioredoxin, 15 μM thioredoxin reductase (*20*), and 100 mM NADPH in a total volume of 10 μL of solution containing 500 mM NaCl, 50 mM Hepes-NaOH (pH 7.6). Where indicated, an aliquot (5 μL) of this mixture was added to 495 μL of apo-SoxR solution (10 μM) that contained 500 mM NaCl, 50 mM Hepes-NaOH (pH 7.6). The kinetics of reconstitution with thioredoxin were monitored at 414 nm, an absorbance maximum for the SoxR [2Fe-2S] clusters (*15*, *18*), to minimize interference by the absorbance of NADPH.

Measurement of SoxR Transcriptional Activity. SoxR activity was assayed by in vitro transcription using plasmid pBD100 as the template (*10*). The SoxR-dependent *soxS* transcript and the SoxR-independent *bla* transcript were quantified by primer extension analysis using improved oligonucleotide primers (*21*). SoxR activity in vivo was monitored using a *soxS′::lacZ* operon fusion present in single copy (*22*). This $\lambda\Phi$ (*soxS′::lacZ*) fusion was introduced into strains WBP570 (Δ*trxA*), WBP571 (Δ*trxA trxB::*Km), WP892 (Δ*trxA gor*522), and DHB4 (*trxA*$^+$ *trxB*$^+$ *gor*$^+$) by isolating λ lysogens of these strains. The *trxA*, *trxB*, and *gor* genes encode, respectively, thioredoxin-A, thioredoxin reductase, and GSH reductase. Strains DHB4, WBP570, and WBP571 (*23*) and WP892 were kindly provided by Professor J. Beckwith (Harvard Medical School). The expression of *soxS′::lacZ* was measured by assaying β-galactosidase activity in SDS/$CHCl_3$-permeabilized cells as described (*7*).

Reagents. GSH, L-cysteine, D-cysteine, *N*-acetyl-L-cysteine, L-cysteine methyl ester, L-cysteine ethyl ester, L-cysteine-*S*-sulfate, L-cystine, 2-aminoethanethiol, 3-mercaptopropionic acid, dithioerythritol, dithiothreitol, NADPH, and Hepes were obtained from Sigma Co. (St. Louis, MO). Recombinant *E. coli* thioredoxin was purchased from Promega Co. (Madison, WI). *E. coli* thioredoxin reductase was kindly provided by Professor C. H. Williams, Jr. (University of Michigan). Other chemicals were obtained in the purest form commercially available.

RESULTS

Effect of Monothiols on Stability of SoxR [2Fe-2S] Clusters. Reduced SoxR [2Fe-2S] clusters typically have an EPR spectrum with g_x at 1.91, g_y at 1.93, and g_z at 2.01 (Figure 1a). As demonstrated previously (*15*), incubation of purified SoxR with reduced GSH at 37 °C for 30 min in aerobic solution caused the [2Fe-2S] clusters to be completely disrupted (Figure 1b). This disrupting effect of aerobic incubation with a thiol extends to *N*-acetyl-L-cysteine (Figure 1c), L-cysteine methyl ester (Figure 1d), and L-cysteine ethyl ester (Figure 1e).

In view of the foregoing results, it was surprising that incubation with L-cysteine did not disrupt the [2Fe-2S] clusters during aerobic incubation (Figure 1f). We, therefore, performed a kinetic analysis to determine whether L-cysteine affected the disrupting activity of GSH. Figure 2A shows typical kinetics for SoxR [2Fe-2S] cluster disruption in the presence of 1 mM GSH; the reaction was monitored at 332

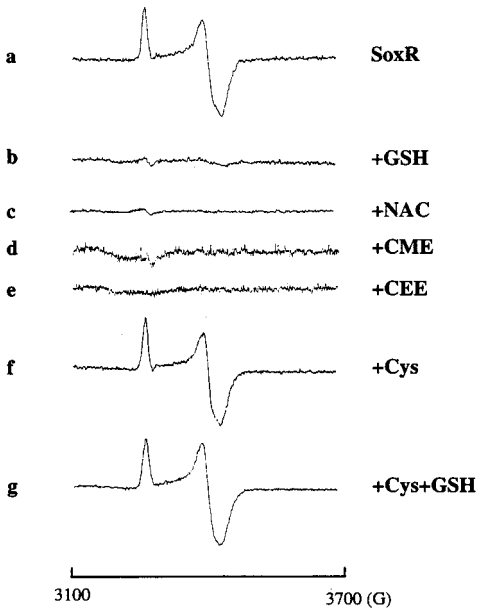

FIGURE 1: EPR spectra of SoxR [2Fe-2S] clusters. Purified SoxR (10 μM) in 500 mM NaCl, 50 mM Hepes (pH 7.6) (trace a) was treated for 30 min at 37 °C with 1 mM GSH (trace b), *N*-acetyl-L-cysteine (NAC; trace c), L-cysteine methyl ester (CME; trace d), L-cysteine ethyl ester (CEE; trace e), L-cysteine (Cys; trace f), or 1 mM GSH and 1 mM L-cysteine (Cys + GSH; trace g). The samples were then reduced with 1 mM freshly prepared sodium dithionite before transfer to EPR tubes. The EPR measurements were as described in Materials and Methods.

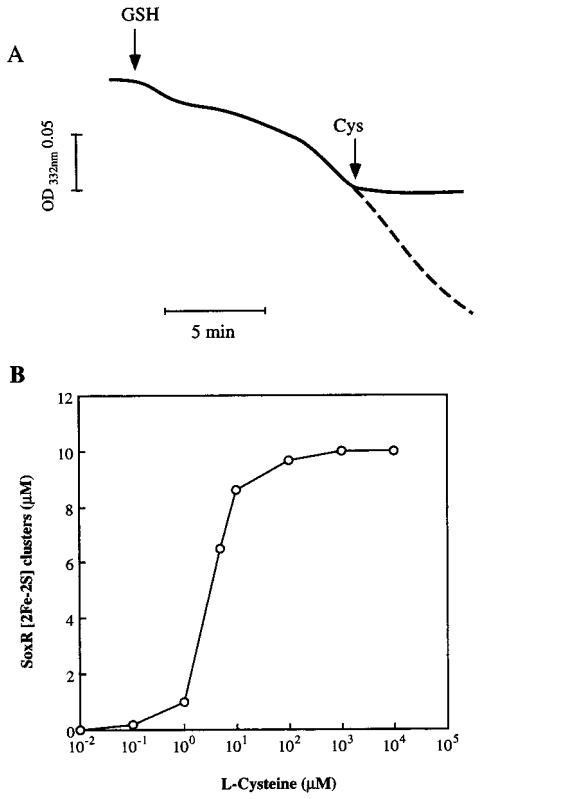

FIGURE 2: Protective effect of L-cysteine against GSH-mediated disassembly of the SoxR [2Fe-2S] clusters. (A) Kinetics of disruption of the SoxR [2Fe-2S] clusters at room temperature. The absorbance of the SoxR [2Fe-2S] clusters (10 μM) was monitored at 332 nm, a maximum of SoxR [2Fe-2S] cluster absorbance (*15*). The decrease in absorbance indicates the disruption of the SoxR [2Fe-2S] clusters. At 13 min after the addition of 1 mM GSH, 1 mM L-cysteine (Cys) was added from a fresh stock of 100 mM (solid line). The dotted line shows the continued disruption of SoxR [2Fe-2S] clusters by GSH in the absence of L-cysteine. (B) Titration of the protective activity of L-cysteine for the SoxR [2Fe-2S] clusters in the presence of 1 mM GSH. Mixtures of 1 mM GSH and L-cysteine at the indicated final concentration were prepared at room temperature, and SoxR was added to a final concentration of 10 μM. The reactions were then incubated at 37 °C for 30 min, and the amount of the SoxR [2Fe-2S] clusters remaining was quantified by EPR spectroscopy as described for Figure 1.

nm, an absorbance maximum of oxidized SoxR [2Fe-2S] clusters (*15*). When L-cysteine was added to the reaction, the loss of absorbance was immediately stopped. This result indicated that [2Fe-2S] cluster disruption by GSH was blocked by L-cysteine, which was confirmed by EPR analysis of the samples after reduction with dithionite (data not shown; see experiments below). If L-cysteine was added prior to the addition of GSH, the SoxR [2Fe-2S] clusters remained intact throughout the incubation at 37 °C for 30 min as shown by EPR analysis (see trace g in Figure 1). Similarly, L-cysteine prevented the disruption of the SoxR [2Fe-2S] clusters by other monothiols such as *N*-acetyl-L-cysteine (data not shown).

L-Cysteine is potent in protecting the SoxR [2Fe-2S] clusters from thiol-mediated disruption. Titration of L-cysteine in reaction mixtures containing 1 mM GSH and 10 μM SoxR [2Fe-2S] clusters showed nearly complete protection by as little as 10 μM L-cysteine, and some protective effect even at 1 μM (Figure 2B).

It is unlikely that L-cysteine binds directly to the SoxR [2Fe-2S] clusters to protect them from disruption by other monothiols, because L-cysteine begins to be effective at a molar ratio of 0.1 relative to SoxR (Figure 2B), and the same concentration dependence for protection by L-cysteine was observed when the SoxR concentration was increased to 20 μM (data not shown). A specific interaction with SoxR might be expected to show stereospecificity, but when L-cysteine was replaced by D-cysteine, the same protective effect against [2Fe-2S] cluster disruption by GSH was observed (data not shown). Finally, the addition of L-cysteine or D-cysteine to SoxR produced no detectable change in the EPR spectrum of the reduced SoxR [2Fe-2S] clusters (Figure 1f,g). Although these experiments do not rule out some type of interaction, L-cysteine could also protect the SoxR [2Fe-2S] clusters by scavenging free radicals in the reaction mixture. Since reactive oxygen intermediates are involved in the GSH-mediated disruption process of the SoxR [2Fe-2S] clusters (*15*), eliminating these reactive oxygen species would block the disruption of the SoxR [2Fe-2S] clusters by GSH.

The dramatic difference between L-cysteine and other monothiols such as GSH, *N*-acetyl-L-cysteine, and L-cysteine methyl or ethyl esters prompted us to examine the effects of still other cysteine derivatives or compounds with structures related to cysteine on the stability of the SoxR [2Fe-2S] clusters. Compounds lacking the cysteine carboxyl moiety (2-aminoethanethiol) or the amino moiety (3-mercaptopropionic acid) lacked the protective activity seen for L-cysteine. These compounds were disruptive in the manner of other monothiols. Under the same experimental conditions, L-cysteine-*S*-sulfate and L-cystine (oxidized L-cysteine) had neither disruptive nor protective effects on the SoxR [2Fe-2S] clusters. Thus, the L-cysteine thiol, carboxyl, and amino groups are essential for the protection of the SoxR

Table 1: Effect of Thiols on SoxR [2Fe-2S] Clusters

thiol effect	thiols	effective concentration
disruptive	GSH	1 mM
	N-acetyl-L-cysteine	1 mM
	L-cysteine methyl ester	1 mM
	L-cysteine ethyl ester	1 mM
	2-aminoethanethiol	1 mM
	3-mercaptopropionic acid	1 mM
protective	L- or D-cysteine	1−10 μM
constructive	dithiothreitol	1 mM
	dithioerythritol	1 mM
	thioredoxin (+thioredoxin reductase and NADPH)	2−8 μM
no effect	oxidized thioredoxin	8 μM
	L-cystine	1 mM
	L-cysteine-*S*-sulfate	1 mM

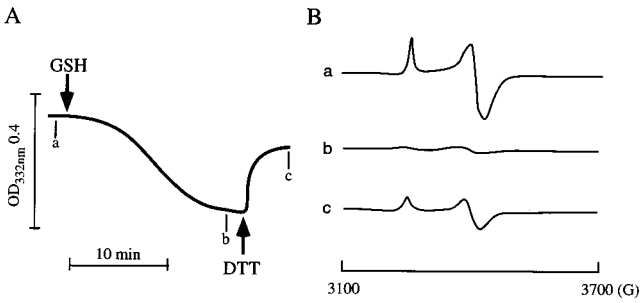

FIGURE 3: Dithiothreitol reverses the disruption of the SoxR [2Fe-2S] clusters by GSH. (A) Kinetics of SoxR [2Fe-2S] cluster disruption by GSH and reformation by dithiothreitol. SoxR (20 μM) containing 500 mM NaCl, 50 mM Hepes-NaOH (pH 7.6) was incubated in an open-top cuvette with 1 mM GSH. The reaction was monitored at 332 nm. After the SoxR [2Fe-2S] clusters were nearly completely disrupted, 1 mM dithiothreitol (DTT) (from a fresh stock solution of 100 mM) was added at the indicated time. (B) EPR measurements of samples taken at the times indicated by a, b, and c in panel A.

[2Fe-2S] clusters from disruption by monothiols such as GSH. Because the redox midpoint potentials of GSH and L-cysteine are very close (*24*), the structure of L-cysteine may have important steric effects in the protective reaction for the SoxR [2Fe-2S] clusters. The effects of the various monothiol compounds and derivatives are shown in Table 1.

Dithiol-Mediated Assembly of the SoxR [2Fe-2S] Clusters. Expanding our investigation showed that bifunctional thiols such as dithiothreitol have a new feature that distinguishes them from monothiols. When dithiothreitol (1 mM) was added to a SoxR reaction in which the [2Fe-2S] clusters had been almost completely disrupted by GSH, the SoxR [2Fe-2S] clusters reappeared rapidly (<2 min) (Figure 3A) with a yield of ~35% of the initial amount of the SoxR [2Fe-2S] clusters, as judged by the amplitudes of EPR spectra of dithionite-reduced samples (Figure 3B). Similar results were observed with dithioerythritol (data not shown).

Dithiothreitol and dithioerythritol are laboratory reagents chosen for their exceptional reducing activity (*25*). We therefore tested whether naturally occurring dithiols can also mediate reformation of monothiol-disrupted [2Fe-2S] clusters in SoxR. With purified apo-SoxR in the absence of Fe^{2+} and S^{2-}, reduced thioredoxin did not produce a significant change in visible absorbance (Figure 4A; the stepwise increase in absorbance is due to the NADPH added with thioredoxin) or in EPR spectroscopy (Figure 5A). When Fe^{2+} and S^{2-}

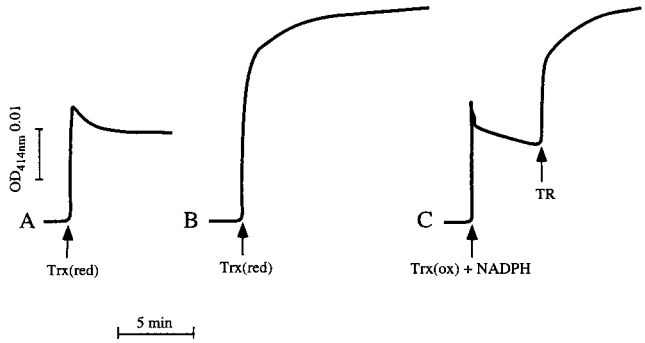

FIGURE 4: Reconstitution of the SoxR [2Fe-2S] clusters by reduced thioredoxin. Apo-SoxR (10 μM) was prepared and purified as described in Materials and Methods. Kinetics were monitored at 414 nm to minimize interference by NADPH absorbance. The reactions were carried out at room temperature under aerobic conditions. (A) Apo-SoxR with reduced thioredoxin (containing 8 μM thioredoxin, 1 mM NADPH, and 0.15 μM thioredoxin reductase); the sharp increase of the absorbance at 414 nm was due to the NADPH. (B) $Fe(NH_4)_2(SO_4)_2$ (80 μM) and Na_2S (40 μM) (both from freshly prepared stock solutions) were added to an apo-SoxR sample before the addition of thioredoxin mixture as in part A. (C) Apo-SoxR containing $Fe(NH_4)_2(SO_4)_2$ (80 μM) and Na_2S (40 μM) was supplemented with thioredoxin (Trx) (8 μM) and NADPH (1 mM); after 4 min, 0.15 μM thioredoxin reductase (TR) was added.

were included in the reaction mixture, reduced thioredoxin promoted the formation of a significant amount of SoxR [2Fe-2S] clusters in <1 min (Figure 4B). The kinetics of SoxR [2Fe-2S] cluster formation were slightly slower when a lower concentration of thioredoxin (2 μM instead of 8 μM) was used in the reaction, but the final amount of SoxR [2Fe-2S] clusters was almost the same (data not shown). Thus, thioredoxin can act catalytically when the reductase is also present. Thioredoxin, thioredoxin reductase, and NADPH were required for the assembly of the SoxR [2Fe-2S] clusters: combination of any two of the three reagents with Fe^{2+} and S^{2-} did not produce the spectroscopic changes characteristic of the SoxR [2Fe-2S] clusters (e.g., see the first part of the reaction shown in Figure 4C).

EPR measurement of the reconstituted SoxR showed that ~75% of the apo-SoxR was converted to SoxR-containing [2Fe-2S] clusters within 5 min after addition of reduced thioredoxin (Figure 5A). In vitro transcription assays showed that such reconstituted SoxR regained its full transcriptional activity (Figure 5B).

In vivo Role for Cellular Dithiols in SoxR Activity. There are at least two thioredoxins and three glutaredoxins in *E. coli* (*23*). Thioredoxins and glutaredoxins are reduced by NADPH, but through different pathways. Reduction of thioredoxin is catalyzed by the flavoenzyme thioredoxin reductase (*20*) using NADPH as a reductant. On the other hand, glutaredoxins are reduced directly by GSH, which in turn is reduced by GSH reductase. GSH reductase is also a flavoenzyme that uses NADPH to reduce oxidized glutathione (*23*).

To test the potential function of dithiol proteins such as thioredoxin in SoxR activation in vivo, we introduced a single-copy operon fusion (*soxS′::lacZ*) into a wild-type strain and into various mutant strains deficient in thioredoxin-A, in thioredoxin reductase, or in the glutaredoxin pathway enzyme GSH reductase (*23*). The expression of *soxS′::lacZ* induced in response to PQ was monitored by measuring

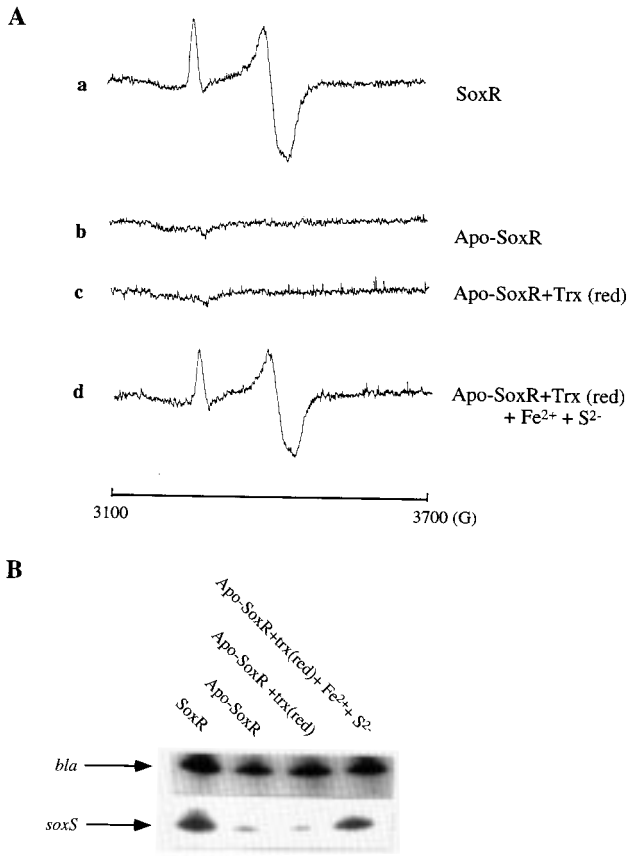

FIGURE 5: Characterization of [2Fe-2S] SoxR reconstituted by thioredoxin. (A) EPR spectra of dithionite-treated SoxR samples. The same amount of SoxR protein (10 μM) was present in each sample: trace a, purified SoxR; trace b, purified apo-SoxR; trace c, purified apo-SoxR incubated for 10 min with reduced thioredoxin as described in Figure 4; trace d, purified apo-SoxR mixed with $Fe(NH_4)_2(SO_4)_2$ (80 μM) and Na_2S (40 μM) before incubating for 10 min with reduced thioredoxin. (B) In vitro transcription assays. Aliquots of the samples shown in panel A were diluted 1000-fold into reactions for in vitro transcriptional activity assays: *bla*, the *bla* gene transcript; *soxS*, the SoxR-regulated *soxS* transcript.

Table 2: Activation of the SoxR-Dependent *soxS* Promoter in Thiol-Deficient Strains[a]

strain	PQ	cell density (OD600)	*soxS′::lacZ* activity (β-galactosidase, Miller units)	induction ratio
DHB4 (WT)	−	0.80	150	
	+	0.78	3490	23
WBP570 (Δ*trxA*)	−	0.58	113	
	+	0.57	3160	27
WBP571 (Δ*trxA*, *trxB::*Km)	−	0.45	116	
	+	0.44	2480	21
WP892 (Δ*trxA*, *gor*522)	−	0.29	390	
	+	0.27	1670	4

[a] Overnight cultures were diluted 1:100 into 5 mL of LB, and grown with shaking at 37 °C for 120 min. Each culture was then divided into two tubes, to one of which was added PQ to 100 μM (+PQ). Incubation and shaking aeration were continued for another 30 min, then β-galactosidase activity was determined as described in **Materials and Methods**. Data shown in the table are the mean values of three experimental results.

β-galactosidase activity (*7, 22*). Although the mutant strains grew more slowly than wild-type *E. coli* (Table 2), no significant difference in SoxR activation was observed for the strains lacking only thioredoxin-A and thioredoxin reductase (Table 2). This result indicated that the SoxR [2Fe-2S] clusters, which are essential for activation of *soxS′::lacZ* expression in response to PQ (*14*), are maintained even in the absence of one of the thioredoxin pathways. It was possible that a role of thioredoxin-A could be substituted by the glutaredoxin pathway (*23*), which is maintained by GSH reductase and GSH. Indeed, a strain lacking both thioredoxin-A and GSH reductase had a much diminished induction ratio in response to PQ, indicative of a defect in maintaining the SoxR [2Fe-2S] clusters (Table 2). Since mutational elimination of GSH from *E. coli* (which should inactivate the glutaredoxin pathways) did not diminish the regulatory activity of SoxR in vivo (*15*), both the thioredoxin and glutaredoxin pathways may help maintain functional [2Fe-2S] clusters in SoxR. Such functional redundancy of the thioredoxins occurs in a variety of pathways, such as the enzymatic cycling of ribonucleotide reductase (*26*).

DISCUSSION

The known biological roles of iron−sulfur clusters have recently been expanded significantly. Iron−sulfur clusters active in electron transfer have been known for some time and now rank in significance alongside other cofactors such as hemes and flavins (*27*). [4Fe-4S] clusters may constitute DNA recognition elements in certain DNA repair proteins (*28*), but recent work also shows that iron−sulfur clusters can fulfill dynamic signaling functions linked to gene expression (*29, 30*). The key examples for these latter roles are two *E. coli* transcription activators: SoxR, which is regulated by the oxidation state of its [2Fe-2S] clusters (*4, 5, 12, 13, 16*); and Fnr, which contains oxygen-sensitive [4Fe-4S] clusters and is active only under anaerobic conditions (*17, 31, 32*). Conversion of the Fnr [4Fe-4S] clusters to [2Fe-2S] clusters, and possibly their disassembly, seems to govern Fnr transcriptional activity (*33*).

The properties of Fnr suggest that disruption or disassembly of iron−sulfur clusters could have general regulatory functions. Indeed, the enzyme biotin synthase also undergoes interconversion of two [2Fe-2S] clusters to one [4Fe-4S] cluster under some conditions (*34*). More generally, the assembly and disassembly of iron−sulfur clusters can occur in vivo without protein degradation. Exposure of *E. coli* to hyperbaric oxygen results in disruption of the [4Fe-4S] clusters of dihydroxy-acid dehydratase, which reappear without new protein synthesis when normal aerobic conditions are restored (*35*). Turnover of such oxidant-sensitive [4Fe-4S] clusters may contribute significantly to the in vivo pool of intracellular "free" iron that potentiates oxidative damage (*36, 37*).

For the SoxR protein, the ready disruption of its [2Fe-2S] clusters upon aerobic exposure to some thiols (*15, 18*, this work) and the stability and high-affinity DNA binding activity of apo-SoxR (*18*) suggest that cluster disassembly could exert a regulatory function under some circumstances. The diminished responsiveness to oxidative stress of SoxR in cells deficient in the dithiol proteins and GSH-dependent glutaredoxins illustrates one situation that supports such an in vivo role. The slightly elevated basal expression of SoxR transcriptional activity in that case may be further evidence for an accumulation of apo-SoxR in the *trxA*$^-$ *gor*$^-$ strain: mutant SoxR proteins with individual cysteines substituted

by alanine lack the [2Fe-2S] clusters in vivo and do not respond to PQ, but do exhibit slightly increased basal activity (*14*).

In addition to a possible decrease in dithiol-mediated assembly of SoxR [2Fe-2S] clusters in the mutant deficient in thioredoxin-A and GSH reductase, elevated levels of oxidized glutathione could also contribute to cluster disruption in the cells, as has been seen in vitro (*15*). A more systematic investigation of strains lacking other dithiols (thioredoxin-B and the three glutaredoxins) would be warranted to characterize their in vivo roles further, although not all possible combinations of mutations have been achieved, and severely deficient cells may not be viable (*23, 26*).

The very effective protection of SoxR [2Fe-2S] clusters shown by L-cysteine is consistent with a role in which this thiol eliminates a key reactive species involved in cluster disassembly. This reactive species might be either a reactive form of SoxR itself generated during the disassembly reaction, or a low-molecular-weight free radical, perhaps derived from GSH as we have proposed (*15*). Other work has shown that cysteine can act as a "sink" for free radicals (*38*), and L-cysteine-based free radicals that may be formed during scavenging evidently do not disrupt the SoxR [2Fe-2S] clusters. If L-cysteine interacts specifically with a SoxR intermediate formed in the thiol-mediated disruption process, such an interaction would also be achieved by D-cysteine (Table 1). Understanding the nature of the SoxR-based intermediates in the thiol-mediated disassembly process and understanding the reaction target(s) of L-cysteine are important goals that will require considerable additional investigation.

The assembly of iron−sulfur clusters in proteins has been studied by several groups (*21, 32, 39*). This process can occur spontaneously, but it is greatly accelerated by NifS protein from *Azotobacter vinelandii*, which produces inorganic sulfide from L-cysteine (*39*). A NifS-like protein has also been isolated from *E. coli* (*41*), and the recently completed *E. coli* genome sequence (*42*) reveals several genes encoding NifS homologues (*43*). The ability of low-molecular-weight dithiols and dithiol proteins such as thioredoxin to promote the assembly of [2Fe-2S] clusters is a newly described phenomenon. The mechanism by which these reactions occur remains unknown, although it could involve reduction of disulfides formed between iron-liganding cysteines in apo-SoxR. If so, we must consider the competitive reactions of protein disulfide reduction and the formation of destructive thiol-based radicals (*15*). If these are the important pathways, then the intramolecular dithiols would favor the former, while intermolecular reactions of monothiols would favor the latter. To pursue this point further, one could propose that reactive monothiols form dead-end mixed disulfides with SoxR cysteines. It may be possible to identify such products.

Although reduced thioredoxin promotes the assembly of SoxR [2Fe-2S] clusters, the in vivo function of thioredoxin could not be easily addressed. Despite the observation that SoxR activation in response to PQ occurred almost normally in a thioredoxin-deficient strain, other dithiol proteins such as glutaredoxin could substitute for thioredoxin functions (*23*). Indeed, redundant functions of these proteins operate in maintaining ribonucleotide reductase activity (*26*) or preventing the formation of disulfides in cytosol (*23*). An alternative possibility is that thioredoxin aids the formation of [2Fe-2S]-containing SoxR in vivo, but that sufficient active protein is produced in its absence to support full activation of *soxS* transcription. The deletion of both thioredoxin-A and GSH reductase seems to have a dramatic effect on SoxR activation in response to PQ in *E. coli*. It could be that the amount of cellular dithiols is decreased to a critical level in this double mutant, such that they could not support full assembly of SoxR [2Fe-2S] clusters.

As summarized in Table 1, there are three thiol classes with respect to their action on SoxR. Thus, the ratio of various thiols may regulate redox signaling through the SoxR [2Fe-2S] clusters by modulating the amount of the [2Fe-2S]-containing protein available. The unexpected destructive and constructive activities of thiols on [2Fe-2S] clusters in SoxR (Table 1) could extend to other proteins and other types of iron−sulfur clusters.

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