Glutathione-mediated destabilization *in vitro* of [2Fe–2S] centers in the SoxR regulatory protein

(oxidative stress/gene transcription/thiol free radical)

HUANGEN DING AND BRUCE DEMPLE*

Department of Molecular and Cellular Toxicology, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115

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ABSTRACT SoxR is a transcription factor that governs a global defense against the oxidative stress caused by nitric oxide or excess superoxide in *Escherichia coli*. SoxR is a homodimer containing a pair of [2Fe–2S] clusters essential for its transcriptional activity, and changes in the stability of these metal centers could contribute to the activation or inactivation of SoxR *in vivo*. Herein we show that reduced glutathione (GSH) in aerobic solution disrupts the SoxR [2Fe–2S] clusters, releasing Fe from the protein and eliminating SoxR transcriptional activity. This disassembly process evidently involves oxygen-derived free radicals. The loss of [2Fe–2S] clusters does not occur in anaerobic solution and is blocked in aerobic solution by the addition of superoxide dismutase and catalase. Although H₂O₂ or xanthine oxidase and hypoxanthine (to generate superoxide) were insufficient on their own to cause [2Fe–2S] cluster loss, they did accelerate the rate of disassembly after GSH addition. Oxidized GSH alone was ineffective in disrupting the clusters, but the rate of [2Fe–2S] cluster disassembly was maximal when reduced and oxidized GSH were present at a ratio of $\approx 1:3$, which suggests the critical involvement of a GSH-based free radical in the disassembly process. Such a reaction might occur *in vivo*: we found that the induction by paraquat of SoxR-dependent *soxS* transcription was much higher in a GSH-deficient *E. coli* strain than in its GSH-containing parent. The results imply that GSH may play a significant role during the deactivation process of SoxR *in vivo*. Ironically, superoxide production seems both to activate SoxR and, in the GSH-dependent disassembly process, to switch off this transcription factor.

Most known proteins that contain iron–sulfur clusters employ these metal centers for electron-transfer, hydratase reactions, or for structural purposes (1). The gene-regulatory functions of iron–sulfur proteins have been described only recently (2). One clear example for such a regulatory role occurs in the *Escherichia coli* protein SoxR (3), which is a homodimer of 17-kDa subunits containing a pair of [2Fe–2S] clusters (4, 5). The metal centers appear to be anchored by a cluster of four cysteine residues located near the C terminus of the SoxR polypeptide (T. Bradley, E. Hidalgo, H.D., and B.D., unpublished data). SoxR *in vivo* governs the induction of more than 12 genes in response to superoxide stress or nitric oxide (6). Preexisting SoxR protein is rapidly converted (<10 min) by these agents into a potent transcriptional activator of the *soxS* gene (7, 8), whose protein product then elevates transcription of many antioxidant and other defense genes (9–12). After withdrawal of the activating signal, induced *soxS* transcription decays with a half-life of ≤ 40 min (13). *In vitro*, SoxR specifically binds to the *soxS* promoter region and activates transcription by σ^{70} -containing RNA polymerase (3–6). However, apo-SoxR (lacking the [2Fe–2S] clusters) binds the *soxS* promoter with unchanged affinity but does not stimulate

transcription (3, 4). Footprinting studies indicated specific structural distortions caused by Fe–SoxR but not by apo-SoxR (3, 4), which suggests that SoxR might stimulate transcription by an allosteric mechanism analogous to that proposed for the homologous MerR protein (14, 15).

The foregoing suggests that the SoxR [2Fe–2S] clusters may constitute the actual sensor of free-radical-induced stress (3–6). We have therefore investigated the assembly and disassembly processes of the [2Fe–2S] clusters in SoxR under physiological conditions. The [2Fe–2S] clusters can assemble spontaneously in SoxR, but this process is strongly accelerated by the *Azotobacter vinelandii* NifS protein to produce fully active Fe–SoxR (16). To our knowledge, the factors affecting disassembly of the SoxR [2Fe–2S] clusters have not been studied systematically. However, apo-SoxR is obtained when 2-mercaptoethanol is included in the protein purification buffers (3). In examining the effects of other thiols, we have now found unexpectedly that reduced glutathione (GSH) in aerobic solution effectively disrupts the [2Fe–2S] clusters to inactivate SoxR *in vitro*. Our studies suggest a role for GSH-based free radicals in this disassembly process, and we show that GSH could also play such an inactivating role *in vivo*.

MATERIALS AND METHODS

SoxR Protein. Purification of SoxR protein from *E. coli* containing the expression plasmid pKOR was performed as described (3), except that the heparin-agarose columns (Life Technologies, Grand Island, NY) loaded with SoxR protein were extensively washed with 350 mM NaCl/50 mM Hepes-NaOH, pH 7.6, prior to elution of SoxR. The purity of SoxR protein in these experiments was $>90\%$, as judged by staining of SDS/polyacrylamide gels.

Transcriptional Activity of SoxR. SoxR activity was assayed by *in vitro* transcription using plasmid pBD100 as the template. The SoxR-dependent *soxS* transcript and the SoxR-independent *bla* transcript were quantified by primer-extension analysis (4). SoxR activity *in vivo* was monitored using a *soxS'::lacZ* operon fusion present in single copy. The $\lambda\Phi$ (*soxS'::lacZ*) fusion was introduced into strains JTG10 (GSH-deficient) and AB1157 (parent of JTG10) (17) by isolating λ lysogens of these strains (6). The expression of *soxS'::lacZ* was measured by assaying β -galactosidase activity (7).

Spectroscopy. A UV/visible spectrophotometer (Perkin-Elmer Lambda 3A) was used to measure the absorbance spectrum of SoxR protein and to monitor the absorbance change as a function of time. The X-band EPR spectra of SoxR were obtained using a Bruker model ESP-300 equipped with an Oxford Instruments 910 continuous flow cryostat (courtesy of J. Stubbe's laboratory, Massachusetts Institute of Technol-

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Abbreviations: Apo-SoxR, SoxR protein without [2Fe–2S] clusters; Fe–SoxR, SoxR protein that contains [2Fe–2S] clusters; GSH, reduced glutathione; GSSG, oxidized glutathione; SOD, superoxide dismutase. *To whom reprint requests should be addressed. e-mail: demple@mbcrr.harvard.edu.

ogy). 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 mT to 370 mT; sample temperature, 20 K; receiver gain, 10^5 .

Assay for Fe Released from [2Fe–2S] Cluster. A 40-fold excess of α, α' -dipyridyl was added to solutions containing SoxR. The absorption of the $[\text{Fe}(\alpha, \alpha'\text{-dipyridyl})_3]^{2+}$ complex at 520 nm [$\epsilon_{520} = 5.2 \text{ mM}^{-1}\text{cm}^{-1}$ (ref. 18)] was measured to determine the concentration of released Fe^{2+} .

Reaction Conditions. All reaction mixtures with SoxR contained 50 mM Hepes (pH 7.6) and 500 mM NaCl. Anaerobic conditions were achieved by continuous flow of argon through a sealed vial (5 ml; final atmospheric O_2 concentration $<0.1\%$). All solutions used for the anaerobic reactions were preequilibrated with argon for at least 20 min.

Chemicals. GSH and oxidized glutathione (GSSG), xanthine oxidase, hypoxanthine, superoxide dismutase (SOD) from bovine erythrocytes, and catalase from bovine liver were purchased from Sigma. Other chemicals were obtained in the purest forms commercially available.

RESULTS

Effect of GSH on the SoxR [2Fe–2S] Clusters *in Vitro*. SoxR protein was expressed and purified as described (3). SoxR protein purified in this way contains per monomer one [2Fe–2S] cluster in the oxidized state and is active in stimulating transcription of the *soxS* gene *in vitro* (3, 4). The visible absorption spectrum of purified SoxR exhibits four distinct absorbance maxima at 332 nm, 414 nm, 462 nm, and 548 nm (Fig. 1A, upper trace), which is a typical spectrum for oxidized [2Fe–2S] clusters in proteins (18). After incubation with 1 mM GSH at 37°C for 30 min in aerobic solution, the spectrum was dramatically changed to a profile (Fig. 1A, lower trace) reminiscent of that for the apoprotein (3). After reduction with dithionite, the control sample showed the expected near-axial helium-temperature EPR spectrum (4, 5), while the GSH-treated protein was EPR-silent (data not shown).

Incubation of a $5 \mu\text{M}$ sample of Fe–SoxR with $200 \mu\text{M}$ 2,2'-dipyridyl for 60 min yielded only $0.1 \pm 0.05 \mu\text{M}$ Fe–dipyridyl complex, which indicates that the [2Fe–2S] clusters in SoxR are stable in the presence of this iron chelator. After Fe–SoxR was treated aerobically with 1 mM GSH at 37°C for 30 min, about 2 equivalents of Fe^{2+} per SoxR monomer were detected in solution ($12.1 \pm 1.0 \mu\text{M}$ Fe–dipyridyl complex). SDS/polyacrylamide gel electrophoresis of SoxR showed no significant change in electrophoretic mobility after GSH treatment (data not shown). These results indicate that the [2Fe–

2S] clusters of SoxR were disrupted by GSH in aerobic solution.

Kinetics of Disruption of the [2Fe–2S] Clusters and Deactivation of SoxR by GSH. The kinetics of the GSH-dependent disruption process of the SoxR [2Fe–2S] clusters were followed by monitoring the absorbance of SoxR at 332 nm, which exhibits a dramatic change upon GSH treatment (Fig. 1B). Monitoring at the other visible absorbance peaks yielded very similar kinetics (data not shown).

Samples of $400 \mu\text{l}$ were removed from a GSH-containing reaction mixture at the times indicated in Fig. 1B and frozen immediately after the addition of sodium dithionite (1 mM, final concentration) to reduce the SoxR [2Fe–2S] clusters for EPR measurements. These spectra (Fig. 1C) showed a loss of the EPR signal that parallels the loss of visible absorbance. The amplitude of the [2Fe–2S] EPR signal at the g_y resonance, which reflects the amount of the [2Fe–2S] cluster present, was linearly related to the absorbance change at 332 nm (Fig. 1D). This correlation validates the use of the 332-nm absorbance to follow this reaction.

Using a primer-extension method to monitor SoxR transcriptional activity *in vitro* (4) demonstrated the inactivation of SoxR in parallel with the loss of visible absorbance and EPR-detectable [2Fe–2S] clusters. The SoxR-dependent transcription of *soxS* gradually decreased and reached a minimum (similar to that of apo-SoxR) after a 30-min incubation with 1 mM GSH, while the SoxR-independent transcription of the *bla* gene was not significantly affected by GSH (Fig. 2).

Role of Reactive Oxygen Intermediates. The kinetic analysis of the GSH-dependent loss of [2Fe–2S] clusters from SoxR revealed an initial lag phase (Fig. 1B), the length of which was found to vary with the protein preparation and to decrease with increasing temperature over the range from 25 to 37°C . These observations suggested a role for uncontrolled components of the reaction such as oxygen. In fact, when Fe–SoxR was incubated with 1 mM GSH anaerobically, the EPR-detectable [2Fe–2S] cluster was completely preserved (Fig. 3). In keeping with the apparent physical stability of the SoxR [2Fe–2S] clusters under anaerobic exposure to GSH, the transcriptional activity of the protein was also maintained (Fig. 3). Thus, oxygen is essential for the destabilization reaction.

The requirement of oxygen for the GSH-dependent destabilization of the SoxR [2Fe–2S] cluster invites a clarification of whether reactive oxygen derivatives are involved in the disruption process. We explored this possibility by adding SOD or catalase to reaction mixtures of SoxR with GSH. The addition of either enzyme strongly inhibited the loss of the characteristic [2Fe–2S] absorbance at 332 nm and combining these two scavenging enzymes almost completely blocked the disruption process (Fig. 4B and C). A small amount of SOD activity was

FIG. 1. Disruption of SoxR [2Fe–2S] clusters by GSH in aerobic solution. Samples of Fe–SoxR ($10 \mu\text{M}$) were incubated with or without 1 mM GSH as indicated in the text. (A) Absorption spectroscopy in the visible range. Traces: upper, untreated Fe–SoxR; lower, after a 30-min incubation at 37°C with GSH. (B) Kinetics of reaction with GSH. The absorbance of Fe–SoxR at 332 nm was monitored as a function of time after addition of 1 mM GSH at 32°C . The small arrows indicate times when $400\text{-}\mu\text{l}$ samples were withdrawn and sodium dithionite was added to a final concentration of 1 mM, and the samples immediately frozen in EPR tubes. (C) EPR spectroscopy. The time point samples indicated in B were analyzed by EPR as described in the text. (D) Linear correlation between absorbance at 332 nm and the amplitude of the g_y signal in the EPR spectrum to reduce SoxR [2Fe–2S] clusters ($R^2 = 0.956$).

FIG. 2. Effect of GSH on the transcriptional activity of SoxR. Fe-SoxR (10 μ M) was treated with GSH at 37°C, and samples were withdrawn at the indicated times and diluted 1:40 with the same buffer without GSH. The diluted samples (1- μ l aliquots) were assayed for SoxR transcriptional activity as described in the text. Untreated Fe-SoxR (indicated by C) and apo-SoxR were used as controls. A similar inactivation of Fe-SoxR by GSH was observed in five experiments, but the amount of *bla* transcript did not vary significantly as a function of GSH treatment.

present in the catalase preparation, but at this level (≤ 10 units/ml) failed to produce the inhibition seen with catalase alone (data not shown). The effects of H_2O_2 or superoxide appear to be indirect. The addition of up to 500 μ M H_2O_2 or of the superoxide-generating enzyme xanthine oxidase acting on 100 μ M hypoxanthine had only a minor effect on the 332-nm absorbance of the SoxR [2Fe-2S] clusters in the absence of GSH (Fig. 4 D and E). Nevertheless, the subsequent addition of GSH to these reaction mixtures disrupted the SoxR [2Fe-2S] clusters even more rapidly than did GSH alone and

FIG. 3. Requirement of oxygen for GSH-dependent disruption of SoxR [2Fe-2S] clusters. Incubations were performed as for Fig. 1A except that some samples were incubated under an argon atmosphere (anaerobic). (Upper) The amount of EPR-detectable [2Fe-2S] clusters present (assayed as the g_y signal amplitude; see Fig. 1). (Lower) SoxR transcriptional activity. Samples: 1 and 2, anaerobic incubation; 3 and 4, aerobic incubation; 2 and 4, incubated with 1 mM GSH; 1 and 3, without GSH; 5, control with apo-SoxR.

eliminated the lag seen in their absence (Fig. 4 A, D, and E). These results indicated that a product of GSH oxidation by H_2O_2 or superoxide leads to disruption of the [2Fe-2S] clusters of SoxR.

Possible Role for GSH Free Radicals. GSSG is readily generated in the presence of H_2O_2 or superoxide (19), but replacement of GSH with GSSG in reactions with Fe-SoxR had no disruption effect on the [2Fe-2S] clusters during incubations of up to 1 h (data not shown). This result suggested that another oxidative product of GSH could be involved, such as a GSH-derived free radical.

Thermodynamically, the amount of GSH free radicals in solution will be maximal when the ratio of GSH to GSSG is close to 1:1 (20). The amount of EPR-detectable [2Fe-2S] clusters in SoxR was measured after incubation in reaction mixtures containing various ratios of GSH to GSSG. The maximum rate of disruption of SoxR [2Fe-2S] clusters was seen when the ratio of GSH to GSSG in solution was $\approx 1:3$ (Fig. 5). This result is consistent with the possibility that a GSH-derived free radical is rate-limiting for disruption of the SoxR [2Fe-2S] clusters.

SoxR Activation in a GSH-Deficient Mutant. We examined whether the inactivating effects of GSH on SoxR *in vitro* are paralleled *in vivo*. The *in vivo* activity of SoxR was monitored by assaying β -galactosidase activity expressed from a single-copy *soxS'::lacZ* operon fusion (6) in cells treated with the redox-cycling agent paraquat to generate intracellular superoxide as an activating signal (7, 8). After addition of 100 μ M paraquat, *soxS'::lacZ* expression in the GSH-deficient mutant JTG10 (17) was substantially higher at all times than expression in its wild-type parent AB1157 (Fig. 6). This difference in induction rates and extents was observed over a range of paraquat levels from 10 μ M to 500 μ M, with a maximal difference of ≈ 4 -fold (Fig. 6 and data not shown). We also note that the basal level of SoxR activation (*soxS'::lacZ* expression in the absence of paraquat) was ≈ 2 -fold higher in the GSH-deficient strain than in the wild type (Fig. 6). Thus, GSH *in vivo* counteracts SoxR activation by free radical stress.

DISCUSSION

Two thiols have now been shown to lead to degradation of the [2Fe-2S] centers of SoxR: 2-mercaptoethanol (3, 4) and, shown here, the biological compound GSH. However, such a disrupting effect is not a universal property of thiol compounds: although *N*-acetylcysteine, a compound related to GSH, causes a similar disassembly of the SoxR [2Fe-2S] clusters, L-cysteine blocks the disassembly, and the dithiols dithiothreitol and dithioerythritol block and partially reverse the disruption process caused by GSH (unpublished data). A biological counterpart of these dithiols might be thioredoxin, which seems to have similar protective effects (unpublished data).

The intracellular GSH concentration (1 to 10 mM; refs. 17, 21, and 22) is similar to the level employed in our experiments. The *in vivo* roles of GSH now seem to be further expanded with the addition of its possible destabilizing effect on a regulatory iron-sulfur center, which seems to limit the extent of SoxR activation. GSH plays a clear role in *E. coli* as a defense against the toxicity of superoxide-generating agents (23) and halogenated compounds (24), making it unlikely that the disrupting effect of GSH on [2Fe-2S] clusters represents the sole selective advantage in this case. Nevertheless, cells could utilize GSH-based free radicals to reverse the activation of SoxR protein by disrupting its [2Fe-2S] clusters. Oxidation-reduction of the SoxR [2Fe-2S] centers may also play a regulatory role, perhaps by influencing the stability of the metal centers (for a detailed discussion, see refs. 4 and 16). The detailed mechanism of SoxR activation remains unknown but certainly does not require GSH (Fig. 6).

FIG. 4. Role of H_2O_2 and superoxide in disruption of SoxR [2Fe-2S] clusters. Aerobic incubations of 20 μM Fe-SoxR at 32°C were monitored by absorbance at 332 nm. Large arrows indicate the time of addition of GSH to a final concentration of 1 mM. In B and C, SOD (100 units/ml) and catalase (Cat., 600 units/ml) were added at the indicated times after GSH addition. In D, H_2O_2 was added at the time indicated to a final concentration of 500 μM , followed after 7 min by 1 mM GSH. In E, xanthine oxidase (0.2 milliunits/ml) and hypoxanthine (100 μM) were added at the time indicated by O_2^- , followed after 7 min by 1 mM GSH.

The blocking ability of GSH against the activation of SoxR *in vivo* was not expected, because previous studies using two-dimensional electrophoretic analysis of *soxRS*-regulated proteins did not reveal a significant effect of GSH (23). At least two experimental details could account for these differences. First, a high paraquat concentration (1 mM) was used in the previous experiments. Moreover, the expression of *soxRS*-regulated proteins is probably not directly proportional to the activity level of SoxR, because such expression lies three steps further downstream (following *soxS* transcriptional activation, SoxS synthesis, and transcriptional activation of the individual target genes) (7, 8).

The mechanism by which GSH leads to disassembly of the SoxR [2Fe-2S] centers appears to be complex. The blocking effects of adding SOD and catalase suggest that the involvement of oxygen in the disassembly reaction may be to supply superoxide or hydrogen peroxide, which greatly accelerate the reaction when supplied directly in the presence of GSH. The disassembly process is also blocked by L-cysteine or ascorbate (unpublished data), each of which can act as a radical scavenger (25). However, we cannot rule out a role for O_2 in other steps of the reaction. The weight of evidence points to a critical role for a radical derived from GSH: (i) GSH needs to be present for superoxide or H_2O_2 to have an effect; (ii) GSSG alone does not support the disassembly reaction; and (iii) the rate of the disassembly reaction is maximal when GSH and GSSG are present at nearly equal concentrations. Possible

species formed under our reaction conditions are $\text{GS}^\cdot$, $\text{GSSG}^\cdot$, and $\text{GSO}^\cdot$ (19, 26), with the presence of GSSG accelerating the production of these species in the presence of GSH and O_2 . The site of the initial reaction of the key reactive species on the [2Fe-2S] cluster is also unknown, and an initial reduction of the SoxR [2Fe-2S] centers ($E'_m = -290$ mV; unpublished data) by $\text{GSSG}^\cdot/\text{GSSG}$ [$E'_m = -1500$ mV (ref. 26)] is certainly also possible. One possibility is that a GSH-derived radical attacks one of the cysteine thiolates that anchor the iron-sulfur center to the protein, perhaps leading to the formation of a mixed disulfide with the protein. GSH could replace the protein ligand on the Fe atom, and additional cycles of attack would be sufficient to disrupt all of the Fe-cysteine bonds. Alternatively, the disruption might be initiated by reactions involving the sulfides of the [2Fe-2S] clusters. Considerable additional experimentation will be required to unravel the detailed mechanism of this GSH-dependent disassembly process.

The generality of the GSH-mediated disassembly of iron-sulfur centers is unknown. Various types of clusters ([4Fe-4S], [3Fe-4S], and [2Fe-2S]) and mononuclear centers lacking inorganic sulfide have been described, all in various protein contexts (1, 2), and the reactivities of these various metal centers undoubtedly vary widely. For example, in contrast to its effects on SoxR, GSH has been proposed to stabilize the [4Fe-4S] cluster of *E. coli* aconitase (27). Our observations clearly show that the biological activity of a key regulatory [2Fe-2S] protein is significantly influenced by GSH. The possibility of a destabilizing effect of this important biological thiol on other iron-sulfur proteins now also merits investigation.

FIG. 5. Effect of the GSH/GSSG ratio on the initial rate of SoxR [2Fe-2S] cluster disassembly in aerobic solution. Various mixtures of GSH and GSSG were freshly prepared in the indicated ratios and added to equal volume of 10 μM samples of SoxR such that $([\text{GSH}] + 2[\text{GSSG}]) = 2$ mM, followed by incubation at 37°C for 10 min. After the addition of sodium dithionite (1 mM), the amount of [2Fe-2S] clusters remaining was determined by EPR spectroscopy (measuring the amplitude of the g_y signal).

FIG. 6. Activation of SoxR in wild-type and GSH-deficient *E. coli* by treatment with paraquat. A single-copy *soxS'::lacZ* operon fusion was placed in the chromosomes of the GSH-deficient mutant JTG10 (open circles) and its wild-type parent AB1157 (solid circles). β -Galactosidase activity was followed after the addition to cultures at 37°C of paraquat to a final concentration of 100 μM .

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