Isolation of reductase for SoxR that governs an oxidative response regulon from *Escherichia coli*

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Abstract SoxR is a transcription factor triggered by oxidative stress in *Escherichia coli*. Recent evidence suggests that novel redox regulation couples oxidation state to promoter activation. We have isolated the reductase for SoxR in *E. coli* using an assay of NADPH- and SoxR-dependent cytochrome *c* reductase activity. When the purified protein was incubated in an anaerobic reaction mixture containing SoxR and NADPH, the reduction of [2Fe-2S] cluster of SoxR was observed by optical and EPR spectroscopy. Our results indicate that the purified protein serves as an NADPH-dependent reduction system for SoxR.

Key words: SoxR; Transcriptional activator; Oxidative stress; Redox regulation; NADPH; *Escherichia coli*

1. Introduction

Cells respond to sublethal levels of oxidative stress by coordinately activating batteries of antioxidant genes [1–6]. The molecular signals that activate these multifunctional defense systems have been the objects of considerable interest. In *Escherichia coli*, the *soxRS* regulon mediates an oxidative stress response that protects the cell against superoxide radical (O_2^-) or nitric oxide [7,8]. The *soxRS* response occurs in two stages. First, an intracellular signal of oxidative stress converts existing SoxR protein into a potent transcriptional activator of the *soxS* gene. Second, the resulting increase in SoxS levels triggers expression of the following regulon genes: *sodA*, encoding Mn-SOD; *nfo*, encoding endonuclease IV a DNA repair enzyme; *zwf*, encoding glucose 6-phosphate dehydrogenase.

SoxR, a ferredoxin-like protein, is a homodimer of 17 kDa subunits containing a [2Fe-2S] cluster essential for in vitro transcriptional activation of the *soxS* promoter [9,10]. It seems likely that the FeS center of SoxR is involved in the signal transduction mechanism that links O_2^- or NO stress to gene activation in the *SoxRS* system. However, little is known about the mechanism of activation.

Recently, it has been shown that univalent oxidation and reduction of the [2Fe-2S] clusters regulates SoxR transcriptional activity. The oxidized form of SoxR stimulates in vitro transcription of its target gene *soxS* and its activity is reversibly inactivated by reduction of the [2Fe-2S] clusters [11,12]. This is consistent with the following in vivo analysis of EPR spectra of intact cells overproducing the wild-type SoxR protein [13–15]. SoxR protein was maintained in the reduced state during normal aerobic growth [13,15], whereas constitutively active mutant forms of SoxR were prone to oxidation of the [2Fe-2S] centers in the absence of superoxide-generating agents [13,14]. Moreover, the rapid redox-switching process of SoxR protein in vivo was followed directly on aerobic exposure of the cells to superoxide-generating agents and removal of the oxidative stress [15]. These results suggest that there must be efficient oxidation-reduction pathways for [2Fe-2S] clusters of SoxR in the cellular system. It seems likely that the [2Fe-2S] centers are maintained in the reduced state by enzymatic reduction. However, the redox systems that reduce SoxR in vivo are unknown.

We found that an *E. coli* strain overproducing SoxR has SoxR-enhanced cytochrome *c* reductase activity in the presence of NADPH. The present communication describes purification of the reductase for SoxR utilizing this assay. Here we present direct observation of the reduction of SoxR by the purified enzyme.

2. Materials and methods

2.1. Strains and plasmids

E. coli strain BL21(λΔE3) and plasmid pET-3Xa were obtained from Novagen.

The expression plasmid for *soxR* was constructed by inserting a PCR-generated fragment containing *soxR* [16] into *Bam*HI- and *Nde*I-digested plasmid pET-3Xa, which positioned the *soxR* coding region behind a *tac* promoter.

2.2. Overproduction of SoxR

A 100 ml saturated culture of *E. coli* BL21 (pET-3Xa-*soxR*) was inoculated into 8 l of medium containing ampicillin (50 μg/ml) and incubated at 37°C in a 10 l fermenter (Oriental Biotechnological System LS-10). At an $A_{600} = 0.7$, the culture was cooled to 18°C and 5 ml of a 1 M IPTG solution was added. After the induction the cells were harvested after 24 h at 18°C. The cell paste was washed and resuspended in an equal volume of 10% sucrose, 10 mM Tris-HCl buffer (pH 8.0), dipped into liquid N_2, and stored at −80°C.

2.3. Purification of SoxR

The thawed cell suspension was incubated in a buffer containing 20 mM MOPS/KOH (pH 7.6), 0.2 M KCl, 1 mM dithiothreitol, and 10% glycerol, 0.0025% phenylmethylsulfonyl fluoride, and 0.5 mg/ml egg white lysozyme. After 60 min at 4°C, the suspensions were sonicated for 5 min, and cell debris was removed by centrifugation at $33\,000 \times g$ for 15 min. The supernatant was applied to a P-11 phosphocellulose (Whatman) column, previously equilibrated with 20 mM MOPS/KOH (pH 7.6), 0.2 M KCl, 1 mM dithiothreitol, and 10% glycerol. Elution was performed with 100 ml of the loading buffer containing a linear gradient of 0.2–1.0 M KCl. The eluted fractions with reddish brown color were collected and then dialyzed against 500 ml solution of 20 mM MOPS/KOH (pH 7.6), 0.3 M KCl, 1 mM dithiothreitol, and 10% glycerol. The sample was then applied to a column of heparin-agarose (Pharmacia). The protein was eluted with 20 mM MOPS/KOH (pH 7.6), 0.5 M KCl, 1 mM dithiothreitol, and

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Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; MOPS, 3-(*N*-morpholino)propane sulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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10% glycerol, frozen in liquid N_2, and stored at −80°C until used. Before use SoxR was passed through Sephadex G-25 equilibrated with the same buffer to remove dithiothreitol. The purity of SoxR was analyzed with SDS-PAGE. The concentration of SoxR was determined by using a millimolar extinction coefficient of 12.7 mM^{-1} cm^{-1} at 417 nm [10].

2.4. Assay of NADPH-dependent cytochrome c reductase

The activity of cytochrome *c* reductase was measured in a 1 ml cuvette containing 2–10 μl enzyme solution, 0.5 mM NADPH, 32 μM cytochrome *c*, 0.2–2 μM SoxR, 0.5 M NaCl, and 20 mM sodium phosphate buffer pH 7.4. Upon the addition of NADPH, the reduction of cytochrome *c* was followed at 550 nm and the reaction rate was measured within 30 s. The SoxR-stimulated activity was calculated by subtracting values obtained in the absence of SoxR.

2.5. Preparation of SoxR-Sepharose 4B

A SoxR affinity column was prepared according to Shin and Oshino [17]. To CNBr-Sepharose 4B gel suspension in 5 ml of 0.1 M $NaHCO_3$ containing 0.5 M NaCl, 10 ml of 42 μM SoxR solution, previously dialyzed against 500 ml solution of 0.1 M $NaHCO_3$ and 0.5 M NaCl, was added. The gel mixture was shaken gently overnight at 4°C. During the reaction, the gel became reddish brown. The mother solution was removed, the gel was suspended in 20 ml of 0.5 M ethanolamine containing 0.05 M $NaHCO_3$ and 0.25 M NaCl at pH 8.0. After shaking for another 2 h at 4°C, it was washed with 0.1 M Tris-HCl buffer, pH 7.5, containing 0.5 M NaCl.

2.6. Reduction of SoxR by purified proteins

The reduction process of SoxR was measured by optical absorption and EPR spectra. The samples contained 0.5 mM NADPH, 20–142 μM SoxR, the purified protein 5–12 μg, 2 mM glucose, 0.5 μM glucose oxidase, 0.5 M KCl, 10% glycerol, and 20 mM MOPS/KOH (pH 7.6) in a 2 ml final volume for optical cell and 0.4 ml final volume for EPR tubes, respectively. The samples were deoxygenated in sealed optical cells or EPR tubes by repeated evacuation and flushing with argon. In addition, the solutions contained glucose and glucose oxidase to ensure continued anaerobiosis. The reactions were started by adding NADPH anaerobically. The EPR tubes were frozen in liquid N_2 at times indicated after addition of NADPH. In control, the spectra in the reduced state of SoxR were obtained on the addition of sodium dithionite.

2.7. Spectrophotometric measurements

Optical absorption spectra were measured with a Hitachi U-3000 spectrometer. EPR spectra were measured with a Varian E-line spectrometer (X-band, 9.301 GHz) with 100 kHz field modulation. The measurement conditions were as follows: microwave power 5 mW; modulation amplitude 0.2 mT; sample temperature 77 K.

2.8. Protein determination

Protein concentrations were determined by the method of Lowry et al. [18] using bovine serum albumin as a standard.

3. Results and discussion

3.1. Purification of SoxR-enhanced NADPH-cytochrome c reductase activity

The addition of NADPH to the fractions passed through the P-cellulose column resulted in reduction of cytochrome *c*. The rate of reduction was found to increase with an increase in the concentration of SoxR up to 2 μM, and it kept constant above 2 μM of SoxR. Under this condition, the activity was 2-fold higher than that observed in the absence of SoxR. We also tested the ability of other [2Fe-2S] cluster proteins to stimulate cytochrome *c* reduction. However, bovine adrenodoxin and spinach ferredoxin had no effect (data not shown). In the absence of NADPH or the crude fractions, reduction of cytochrome *c* was not seen. On the other hand, 1 mM NADH could substitute for NADPH, but the activity was about 5-fold lower than that of NADPH. These results raise the possibility that the activity is associated with an NADPH-dependent reduction system for SoxR. Therefore, this activity was further purified, utilizing assay of cytochrome *c* reduction in the presence of 0.5 mM NADPH and 2 μM SoxR.

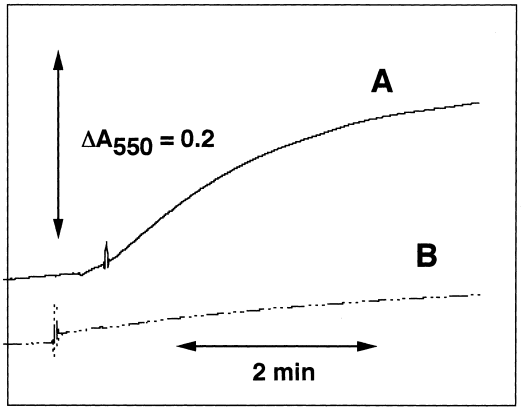


Fig. 1. Absorbance changes of cytochrome *c* at 550 nm on addition of 0.5 mM NADPH to the reaction mixtures containing the eluted fraction from a SoxR affinity column in the presence (A) and absence (B) of 2 μM SoxR. Reactions were performed at 25°C in a total volume of 1 ml containing 1.2 μg purified protein, 32 μM cytochrome *c*, 0.5 mM NADPH, 2 μM SoxR, 0.5 M NaCl, and 20 mM sodium phosphate buffer pH 7.4.

The eluate from the P-cellulose column was dialyzed against a solution of 20 mM Tris-HCl buffer pH 7.4, and was then applied to a column of DE-52 (Whatman) (3×10 cm) previously equilibrated with the same buffer. The column was

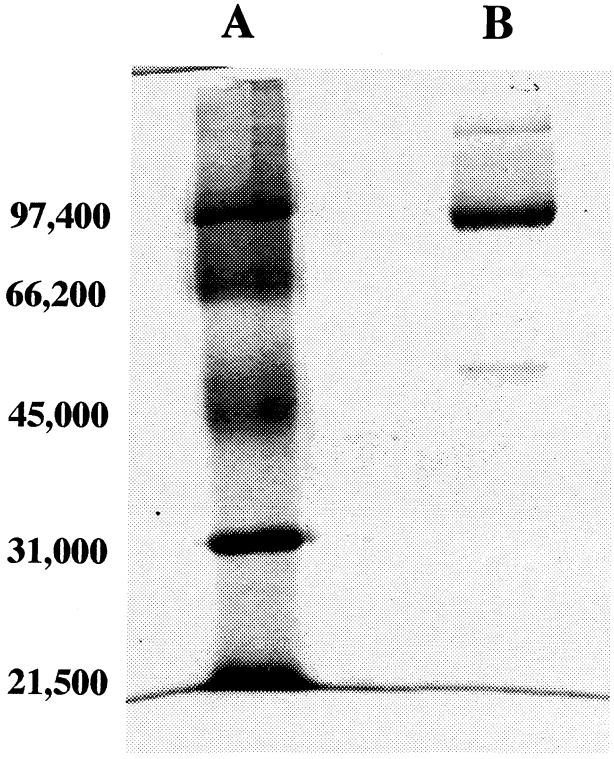


Fig. 2. SDS-PAGE analysis of purified protein. 8% Polyacrylamide was stained with Coomassie blue. Lanes: A, molecular weight markers; phosphorylase b, 97400; bovine serum albumin, 66200; ovalbumin, 45000; carbonic anhydrase, 31000; trypsin inhibitor, 21500; B, 3 μg of purified protein.

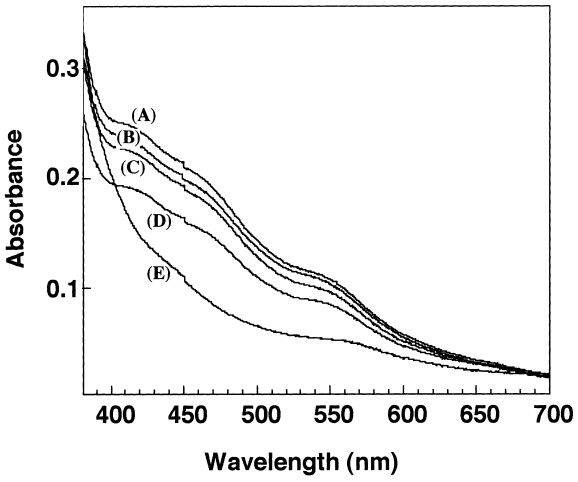


Fig. 3. Spectral changes of SoxR on anaerobic addition of NADPH and the purified protein. The sample contained 5.8 μg purified protein, 0.5 mM NADPH, 18 μM SoxR, 2 mM glucose, 0.5 μM glucose oxidase, 0.5 M KCl, 20 mM MOPS/KOH pH 7.6, and 10% glycerol in 2 ml final volume. The spectra were taken at 0 min (A), 3 min (B), 6 min (C), 30 min (D) after addition of NADPH. Spectrum E was measured on the addition of sodium dithionite.

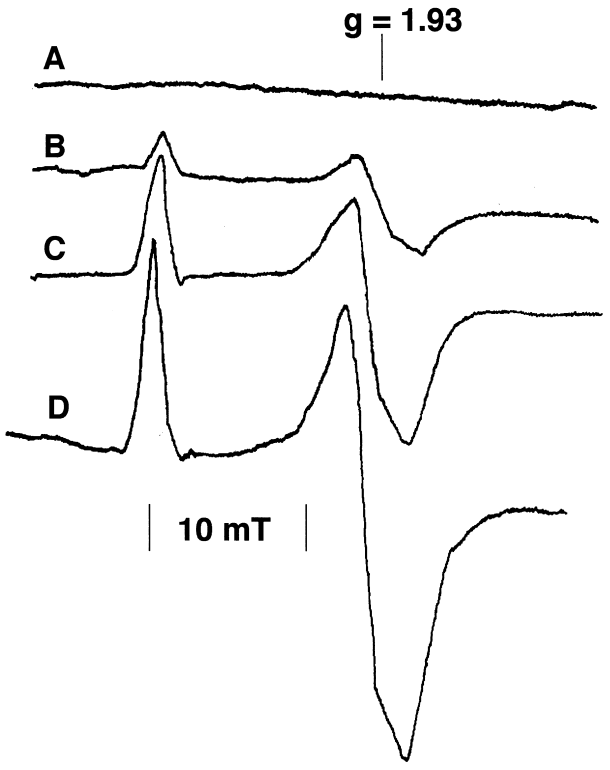


Fig. 4. Changes of EPR signal on anaerobic addition of NADPH and the purified protein. The sample contained 11.6 μg purified protein, 0.5 mM NADPH, 142 μM SoxR, 2 mM glucose, 0.5 μM glucose oxidase, 0.5 M KCl, MOPS/KOH pH 7.6, 10% glycerol in 0.4 ml final volume. The sample tubes were dipped into a Dewar filled with liquid nitrogen at 0 min (A), 10 min (B), 30 min (C) after addition of NADPH, and then EPR spectra were measured. Spectrum D was measured on the addition of sodium dithionite.

washed with the same buffer. The SoxR-stimulated active fractions passed through the column.

The eluate from DE-52 was applied to a 2′,5′-ADP agarose (Pharmacia) column (1×10 cm), previously equilibrated with 20 mM Tris-HCl buffer, pH 7.4. The protein was eluted with 20 mM Tris-HCl buffer pH 7.4 containing 1 mM $NADP^+$, providing a 100-fold purification of enzyme activity in this step. The sample was then applied to a SoxR affinity column (0.7×5 cm) equilibrated with 20 mM Tris-HCl buffer pH 7.4. The activity was eluted with the same buffer containing 0.5 M NaCl. Fig. 1 shows NADPH-cytochrome *c* reductase activity of the eluted fractions. The activity was found to be stimulated about 7-fold by 2 μM SoxR. The active fractions were concentrated by ultrafiltration using an ultrafiltration apparatus (Amicon).

The concentrated sample was injected into a TSK-Gel G 3000 5W column (0.75×60 cm) with a high performance chromatography system (HPLC, Tosoh system). The column was pre-equilibrated with 20 mM sodium phosphate buffer pH 7.4 at a flow rate of 0.5 ml/min. The SoxR-enhanced activity was seen in the fraction with an estimated molecular weight of 85 000, which was determined by gel filtration analysis of protein marker calibration on the same column (data not shown). In the other fractions from the column, the activity was not detected. The active fractions were combined and concentrated by ultrafiltration.

A summary of the results of the purification of the reductase is shown in Table 1. The overall purification affords a 737-fold purification of enzyme activity with 4.3% recovery. The purified enzyme gives a major band of molecular weight of 84 000 and minor bands of molecular weights 50 000 and 150 000 in SDS-PAGE gels (Fig. 2). The molecular weight of the major band is consistent with the result of gel filtration analysis (see above).

3.2. Reduction of SoxR by purified reductase

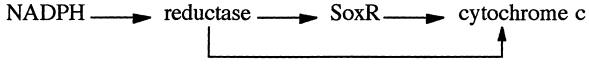
The additions of 0.5 mM NADPH and the purified reductase to a solution of SoxR under anaerobic condition resulted in a bleaching of [2Fe-2S] clusters of SoxR, consistent with reduction of SoxR (Fig. 3). Approximately 50% of SoxR was reduced during 30 min under this condition. When the sample SoxR was aerated, the original spectrum was completely restored within 2 min, indicating that SoxR is readily autooxidized reversibly. In the absence of NADPH or the purified enzyme, the spectral changes due to the reduction of SoxR could not be detected at all.

Table 1
Purification of SoxR-enhanced NADPH-cytochrome *c* reductase

Fraction	Protein (mg)	Specific activity (μmol of cyt *c* min^{-1} mg^{-1})	Total activity (μmol of cyt *c* min^{-1})	Purification fold	Recovery (%)
P-cellulose flow through	2247	0.0194	43.6	1	100
DE-52 flow through	399	0.0677	27.0	3.5	62
2′,5′-ADP agarose eluate	1.6	1.9	3.0	98	6.8
SoxR affinity eluate	0.7	3.6	2.5	185	5.7
Gel filtration	0.13	14.3	1.9	737	4.3

Under similar conditions, EPR spectra were measured. The samples of reaction mixtures in EPR tubes were frozen at the times indicated in Fig. 4. No EPR spectrum was detected in the sample frozen immediately after adding NADPH. At 10 min, an EPR spectrum characteristic of a reduced SoxR [2Fe-2S] cluster was observed. The EPR signal was further increased at 30 min. Comparison of the signal intensity with that obtained by sodium dithionite shows that 50–60% of SoxR protein contained reduced [2Fe-2S] clusters in the sample. In a control experiment, no EPR spectra were detected in the absence of the purified enzyme (data not shown).

In the present study, the isolation of the reductase for SoxR was made possible by the use of an assay monitoring cytochrome *c* reduction. We assume that cytochrome *c* serves as an electron acceptor of SoxR in this assay. In order to test whether the reduced state of SoxR can reduce cytochrome *c*, the addition of equivalent cytochrome *c* to the reduced state of SoxR, which was prepared by the enzymatic reduction (see above), was examined under anaerobic condition. The oxidation of SoxR and the reduction of cytochrome *c* were seen concomitantly (data not shown). In the present assay, therefore, the sequence of electron transfer can be expressed as follows:



In this scheme, the present data shows that the purified reductase can react with not only SoxR but cytochrome *c* directly. However, the rate in the reduction of SoxR is about 100 times larger than that of cytochrome *c*. This is verified by the fact that the rate of cytochrome *c* reduction was enhanced approximately 7–10-fold increase by SoxR, when 1.2 μg purified enzyme, 2 μM SoxR, and 32 μM cytochrome *c* were present in the assay medium.

It is important to note that this is the first direct observation of enzymatic reduction of SoxR. Therefore, we demonstrate that the purified reductase is an NADPH-dependent redox system that reduces SoxR in vivo. This suggests that Fe-S centers are maintained in the reduced state by reactions linked by NADPH, which counteract its autooxidation. If so, the *soxRS* regulon responds to the consequence of a decrease in the ratio of NADPH to $NADP^+$. This is consistent with the finding that SoxR was readily activated in glucose 6-phosphate dehydrogenase-deficient *E. coli* [19]. However, our results do not exclude the existence of other pathways of the reduction of SoxR.

The experiments presented here demonstrate that the purified reductase can catalyze electron transfer from NADPH to [2Fe-2S] clusters of SoxR. A specific interaction between SoxR and the purified reductase seems to be important for the reduction, since this process cannot be explained simply by the redox potentials of [2Fe-2S] centers of SoxR and its electron donor. Gaudu and Weiss [11] reported that the reduced forms of ferredoxin ($E_0 = -380$ mV) or flavodoxin ($E_0 = -450$ mV) [20] did not operate the reduction of SoxR ($E_0 = -283$ mV) [12,13]. Availability of homogeneous enzyme preparations should permit clarification of the mechanism of the reduction. Molecular cloning to ascertain the amino acid sequence of the reductase may permit a detailed comparison with other enzymes.

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