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ARTICLE *in* BIOCHEMISTRY · DECEMBER 2014

Impact Factor: 3.02 · DOI: 10.1021/bi500931w · Source: PubMed

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Binding of Promoter DNA to SoxR Protein Decreases the Reduction Potential of the [2Fe−2S] Cluster

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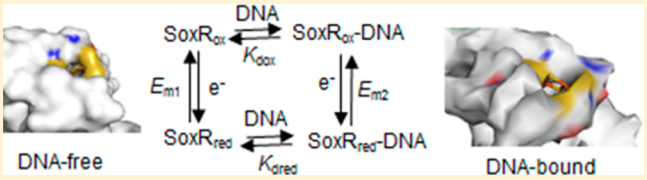
Supporting Information

ABSTRACT: The [2Fe−2S] transcriptional factor SoxR, a member of the MerR family, functions as a sensor of oxidative stress in *Escherichia coli*. The transcriptional activity of SoxR is regulated by the reversible oxidation and reduction of [2Fe−2S] clusters. Electrochemistry measurements on DNA-modified electrodes have shown a dramatic shift in the reduction potential of SoxR from −290 to +200 mV with the promoter DNA-bound [Gorodetsky, A. A., Dietrich, L. E. P., Lee, P. E., Demple, B., Newman, D. K., and Barton, J. K. (2008) DNA binding shifts the reduction potential of the transcription factor SoxR, *Proc. Natl. Acad. Sci. U.S.A. 105*, 3684−3689]. To determine the change of the SoxR reduction potential using the new condition, the one-electron oxidation−reduction properties of [2Fe−2S] cluster in SoxR were investigated in the absence and presence of the DNA. The [2Fe−2S] cluster of SoxR was completely reduced by nicotinamide adenine dinucleotide phosphate (NADPH)−cytochrome P450 reductase (CRP) in the presence of a NADPH generating system (glucose 6-dehydrogenase and glucose-6 phosphate), indicating that CRP can serve as an NADPH-dependent electron carrier for SoxR. The reduction potential of SoxR was measured from equilibrium data coupled with NADPH and CRP in the presence of electron mediators. The reduction potentials of DNA-bound and DNA-free states of SoxR were −320 and −293 mV versus NHE (normal hydrogen electrode), respectively. These results indicate that DNA binding causes a moderate shift in the reduction potential of SoxR.



The [2Fe−2S] transcription factor SoxR, a member of the MerR family, regulates the *soxRS* response to oxidative stress in bacteria.[1,2] Activated SoxR enhances the production of SoxS, a transcription activator, which in turn enhances the production of various antioxidant proteins and repair proteins.[3] The oxidized form of SoxR (SoxR$_{ox}$) stimulates in vitro transcription of its target gene *soxS*, and its activity is reversibly inactivated by reduction of the [2Fe−2S] clusters.[4−6] According to in vivo analysis of EPR spectra of intact cells overproducing the SoxR protein, SoxR is maintained in its reduced inactive form during aerobic growth.[7−9] Upon exposure of *Escherichia coli* to superoxide (O_2^-)-generating compounds such as paraquat and other redox-cycling compounds, the [2Fe−2S] cluster of SoxR is oxidized to the [2Fe−2S]$^{2+}$ active form.[8] The in vivo transcription of *soxS* mRNA correlates with the redox states of SoxR as measured by EPR.[8]

X-ray crystallographic structures of the promoter *soxS* bound and free forms of *E. coli* SoxR in the oxidized state have been determined at resolutions of 2.8 and 3.2 Å, respectively.[10] The SoxR protein consists of a DNA-binding domain, a dimerization helix, and an Fe−S cluster-binding domain. The [2Fe−2S] cluster of SoxR is coordinated by four cysteine residues (Cys-119, Cys-122, Cys-124, and Cys-130), and is nearly completely exposed to the solvent. The structure of the DNA when bound to the oxidized state of SoxR is a bent conformation with local untwisting,[10] similar to that when bound to other MerR family proteins.[11,12] Upon binding of SoxR to the DNA, the DNA-binding domain and the Fe−S cluster domains of SoxR undergo outward rotation, accompanied by a change in the relative positions of the dimerization helices.[10] However, the structural features of [2Fe−2S] cluster of free and DNA-bound forms do not differ significantly. UVRR spectroscopy of Trp98 and Trp91 in SoxR have shown that these tryptophan residues undergo small environmental changes upon DNA binding.[13,14]

Since the transcriptional activity of SoxR is regulated by an alteration of the cellular redox balance, it is important to know the reduction potential of this protein. The reduction potential of SoxR without bound DNA from *E. coli*[5,6] and *Pseudomonas aeruginosa*[15] was determined to be approximately −290 mV, a value close the intracellular reduction potential of *E. coli* (−260 to −280 mV).[16] On the other hand, electrochemistry measurements on DNA-modified electrodes have shown that the reduction potential of SoxR bound to DNA is +200 mV. This potential value corresponds to a dramatic shift of +490 mV versus the values found in the absence of DNA.[17] However, the reduction potential is beyond the range ever previously observed for [2Fe−2S] proteins, which vary from −150 to −460 mV.[18,19] In addition, a very significant difficulty in comparing these values to assess the effect of DNA is that these DNA-bound and DNA-free experiments were carried out under completely different conditions. Understanding the effect of the

Received: July 29, 2014
Revised: November 23, 2014

DOI: 10.1021/bi500931w
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binding of DNA on the redox properties of SoxR requires the same conditions to be used.

NADPH−cytochrome P-450 (CRP) contains one FAD and one FMN per molecule of the enzyme. The FAD accepts the reducing equivalents of two electrons from NADPH to the one-electron acceptor cytochrome P-450 via the FMN domain.[20] The reduction potentials of the individual one-electron couples of FAD and FMN of CRP are −109 mV(FMN/FMNH$^{\bullet}$), −270 mV(FMNH$^{\bullet}$/FMNH$_2$), −290 mV (FAD/FADH$^{\bullet}$), and −365 mV(FADH$^{\bullet}$/FADH$_2$), respectively.[21] This raises the possibility that SoxR may accept an electron from both flavins. It is possible to measure the reduction potential from equilibrium data coupled with NADPH and CRP in the presence of electron-transfer mediators. We report here the determination of the reduction potential of SoxR in the absence and presence of DNA. We have shown, in contrast to the previous report,[17] that the reduction potential in the SoxR is a comparatively small negative shift upon DNA binding.

MATERIALS AND METHODS

Expression and Purification. The expression plasmids for *E. coli* SoxR were transformed into *E. coli* C41(DE3) and coexpressed with the *isc* operon[22] as described previously.[23] SoxR was purified essentially as described previously. SoxR protein samples were purified as the oxidized form[23] and confirmed as >95% homogeneous by SDS−polyacrylamide gel electrophoresis. The concentration of SoxR was determined using an extinction coefficient of 12.7 mM^{-1} cm^{-1} at 417 nm.[24]

SoxR−DNA complexes were prepared according to the method previously used for its crystallization.[23] The palindromic oligonucleotide (5′-G**CCTCAAGTTAACTTGAGG**C-3′, where the *soxS* promoter sequence is in bold), purchased from Sigma Genosis Biotech Co., Ltd. (Japan), formed double-stranded DNA. The oligonucleotide was dissolved in aqueous solution containing 20 mM Tris-HCl (pH 7.6), 50 mM KCl, and 10 mM potassium/sodium tartrate, heated to 94 °C, and gradually cooled to room temperature. For preparation of SoxR−DNA complexes, 40−50 μM SoxR (in a solution also containing 20 mM MOPS, pH 7.6, 300 mM KCl, and 10 mM potassium/sodium tartrate) and the oligonucleotide solution were mixed at molar ratios of 2:1.05−1.1 and incubated for more than 4 h at 4 °C.[23] Below 0.3 M KCl, the complex formation was confirmed by native polyacrylamide gel.[23]

The expression plasmids pCWori^{+} for porcine NADPH−cytochrome P-450 reductase (CRP) were transformed into *E. coli* BL21 as described previously.[25] CRP was purified essentially as described previously.[26] All other chemicals were of the highest grade available from commercial suppliers.

Assay of SoxR Reduction by NADPH/CRP. The reduction of SoxR was measured in a 3 mL cuvette using optical absorption spectroscopy. The samples contained 100−220 μM NADPH, 40−100 μM SoxR, and 0.3 M KCl. Indigodisulfonic acid (E_0' = −116 mV)[27] and phenosafranine (E_0' = −289 mV)[28] were used for as the electron-transfer mediators. The samples were deoxygenated in sealed optical cells by repeated evacuation and flushing with argon. The reactions were started by adding NADPH. The spectra of the SoxR solution were monitored until no further changes were observed, indicating that the solutions had reached equilibrium. The reduction of SoxR was monitored using Δ(absorbance coefficient) (mM^{-1} cm^{-1}) of 5.8 (417 nm) and 7.8 (462 nm) between the oxidized and reduced forms. No absorbance of indigodisulfate and phenosafranine was detected in visible region due to the reduced states of these mediators during the reactions. The concentration NADPH was calculated using extinction coefficient of 6.22 mM^{-1} cm^{-1} at 340 nm.

Optical absorption spectra were measured with a Hitachi U-2900 spectrometer.

RESULTS

The addition of 137 μM NADPH and 0.4 μM CRP to a solution of SoxR under anaerobic conditions resulted in a decrease in absorption of [2Fe−2S] clusters of SoxR from 400 to 600 nm (inset of Figure 1), concomitant with the decrease of

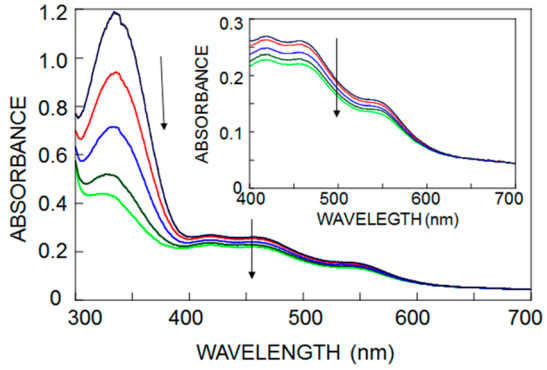

Figure 1. Spectral changes of SoxR upon anaerobic addition of NADPH and CRP. The sample contained 137 μM NADPH, 25 μM SoxR, 0.42 μM CRP, 0.3 M KCl, and 20 mM MOPS/KOH, pH 7.6, in 2 mL final volume. The spectra were taken at 0 (black), 14 (red), 25 (blue), 44 (dark green), and 60 min (light green) after addition of NADPH. The inset shows an expanded scale of absorbance changes of SoxR.

NADPH at 340 nm (Figure 1). When the sample was aerated, the original spectrum was completely restored within 5 min (data not shown), which indicates that the absorbance changes in Figure 1 correspond to reversible reduction of the [2Fe−2S] cluster of SoxR, and not by irreversible destruction of the iron−sulfur cluster. Approximately 10−20% of SoxR was reduced during the first hour and further absorption changes were not observed under the conditions. Upon addition of NADPH generating systems (glucose 6-phosphate and glucose 6-dehydrogenase) to the solution, SoxR was nearly completely reduced (Figure 2). Inset of Figure 2 shows difference spectra obtained after the reaction, where the difference spectrum obtained at 25 min was nearly identical to the SoxR$_{red}$ minus SoxR$_{ox}$ spectrum. Based on these results, CRP can serve as an NADPH- dependent electron carrier for SoxR through protein−protein electron transfer (Figure 3).

Since equilibrium of oxidation−reduction of SoxR and NADPH/CRP was difficult to reach, reduction was carried out in the presence of a small amount of electron-transfer mediators (indigodisulfate and phenosafranine).[21,27,28,30]

$$\mathrm{NADPH} + 2[2\mathrm{Fe}{-}2\mathrm{S}]^{2+} \overset{K_{eq}}{\rightleftharpoons} \mathrm{NADP}^{+} + 2[2\mathrm{Fe}{-}2\mathrm{S}]^{+} + H^{+} \quad (1)$$

The mediators are required for electron transfer between protein molecules. Equilibrium constants for the oxidation−reduction of SoxR and NADPH/CRP (Reaction 1) could be measured, for instance, from the result shown in Figure 4. Approximately 50% of SoxR was reduced during the first 10 min under these conditions. The equilibrium mixture contained

DOI: 10.1021/bi500931w
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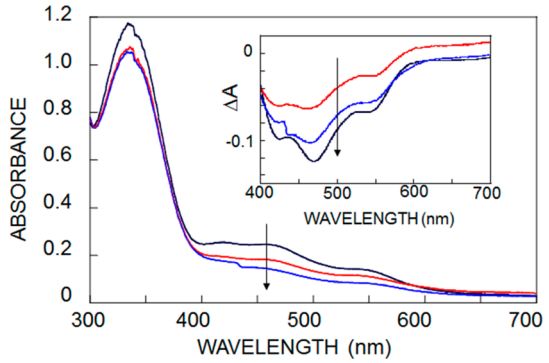

Figure 2. Spectral changes of SoxR upon anaerobic addition of CRP and NADPH along with an NADPH-generating system. The sample contained 137 μM NADPH, 25 μM SoxR, 0.42 μM CRP, and the NADPH generating system (1 mM glucose-6-phosphate and glucose-6-dehydrogenase), 0.3 M KCl, and 20 mM MOPS/KOH, pH 7.6, in 2 mL final volume. The spectra were taken at 0 (black), 7 (red), and 25 min (blue) after addition of NADPH. The inset shows difference spectra before and after the reaction at 7 (red) and 25 min (blue) and oxidized minus reduced SoxR (black).

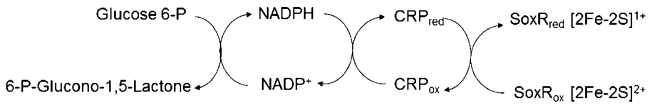

Figure 3. Coupling reactions of NADPH consumption with CRP by SoxR.

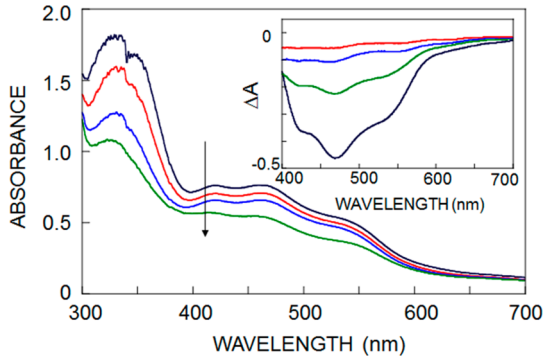

Figure 4. Spectral changes of SoxR upon anaerobic addition of NADPH and CRP in the presence of indigodisulfate and phenosafranine. The sample contained 161 μM NADPH, 75 μM SoxR, 0.42 μM CRP, 1 μM indigodisulfate, 1 μM phenosafranine, 0.3 M KCl, 20 mM MOPS/KOH, pH 7.6, in 2 mL final volume. The spectra were taken at 0 (black), 5 (red), 10 (blue), and 15 min (green) after addition of NADPH. The inset shows difference spectra before and after the reaction at 5 (red), 10 (blue), and 15 min (green) and oxidized minus reduced SoxR (black).

components (NADPH, NADP$^+$, indigodisulfate, phenosafranine, CRP, SoxR$_{ox}$, and SoxR$_{red}$). Since no absorbance other than SoxR was detected in the visible region during the measurements (inset of Figure 4), the concentrations of SoxR$_{ox}$ and SoxR$_{red}$ were estimated. The absorbance correction due to the oxidation of NADPH was subtracted from absorbance changes of SoxR at 340 nm using extinction coefficients (mM^{-1} cm^{-1}) of ε_{ox340} = 20.7 and ε_{red} = 11.0. Their concentrations were estimated on the basis of the absorbance data listed in Table 1. On the assumption that E_0'(NADPH/NADP$^+$) = −340 mV,[29] the data yielded, for instance, E_0'(SoxR$_{red}$/SoxR$_{ox}$) = −293 mV from the Nernst equation (eq 2), where R and F

Table 1. Calculation of the Oxidation−Reduction Potentials of SoxR from the Equilibrated Reactions with NADPH/CRP[a]

composite	NADPH (μM)	NADP$^+$ (μM)	[SoxR]$_{ox}$ (μM)	[SoxR]$_{red}$ (μM)	E_0' (mV)
SoxR	7.2	146.6	29.6	44.4	−293
	7.3	79.3	29.5	3.2	−313
SoxR−DNA	8.2	128.8	29.3	3.4	−319
	24.3	132.6	29.0	2.7	−301

[a]All experiments were carried out in 20 mM MOPS-KOH buffer (pH 7.6) and 0.3 M KCl at 25 °C under an argon atmosphere: CRP 0.42 μM, indigodisulfate 1 μM, phenosafranine 1 μM. The reduction was measured from the decreases of NADPH and SoxR$_{ox}$ in absorbance at 340 and 460 nm, respectively. A value of −340 mV in E_0' (NADPH/NADP$^+$) was used for calculation.

are the gas constant and the Faraday constant, respectively. The value is comparable to that reported previously (−285 mV).[5,6]

$$E_0'(\mathrm{NADPH/NADP^+}) + \frac{RT}{2F}\ln([\mathrm{NADP^+}]/[\mathrm{NADPH}]) = E_0'(\mathrm{SoxR_{red}/SoxR_{ox}}) + \frac{RT}{F}\ln([\mathrm{SoxR_{ox}}]/[\mathrm{SoxR_{red}}]) \quad (2)$$

Experiments under similar anaerobic conditions were carried out with the DNA−SoxR complex. Figure 5 shows the

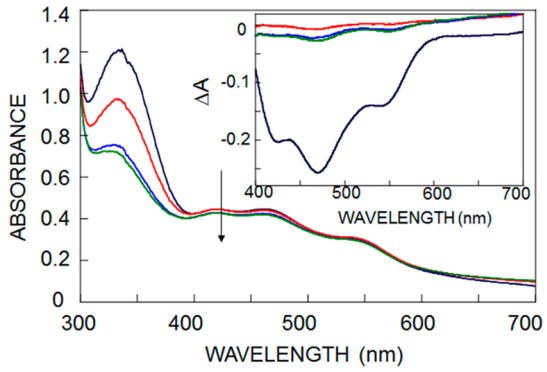

Figure 5. Spectral changes of SoxR−DNA upon anaerobic addition of NADPH and CRP in the presence of indigodisulfate and phenosafranine. The sample contained 160 μM NADPH, 40 μM SoxR, 0.42 μM CRP, 1 μM indigodisulfate, 1 μM phenosafranine, 0.3 M KCl, 20 mM MOPS/KOH, pH 7.6, in 2 mL final volume. The spectra were taken at 0 (black), 5 (red), 10 (blue), and 30 min (green) after addition of NADPH. The inset shows difference spectra before and after the reaction at 5 (red), 10 (blue), and 30 min (green) and oxidized minus reduced SoxR (black).

absorbance changes of SoxR−DNA complex after addition of NADPH. Noted that less than 10% of the [2Fe−2S] was reduced during the first 10 min (inset of Figure 5), and further changes were not observed under these conditions. This suggests that the reduction potential of the DNA−SoxR is more negative than that of DNA-free form. The equilibrium data are shown in Table 1. A value of −310 to −330 mV was calculated for E_0'(SoxR$_{red}$−DNA/SoxR$_{ox}$−DNA). In a control experiment, a similar experiment was performed in the presence of nonsence DNA, but more than 50% of SoxR was reduced under these conditions, as the case of the presence of DNA (Figure S1, Supporting Information).

DOI: 10.1021/bi500931w
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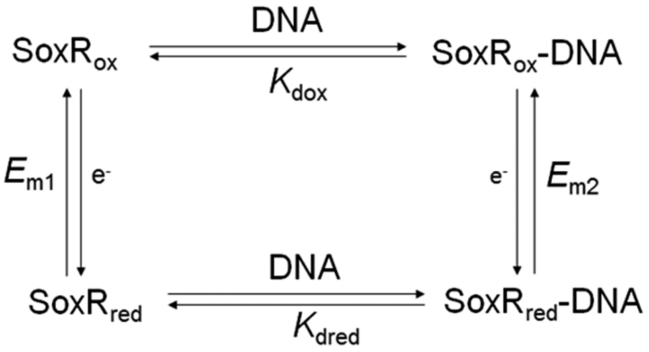
DISCUSSION

Based on the present results, the equilibria presented in Scheme 1 were assumed to occur, where K_{dox} is the dissociation

Scheme 1. Thermodynamic Relationship of the DNA Binding to $SoxR_{ox}$ and $SoxR_{red}$[a]



[a] E_{m1} and E_{m2} are reduction potentials of [2Fe−2S] cluster of SoxR with DNA-free and DNA-bound forms, respectively.

constants of $SoxR_{ox}$ and the DNA and K_{dred} is that between $SoxR_{red}$ and the promoter DNA. It is apparent from the results shown in Figures 4 and 5 that the redox equilibrium between $SoxR_{ox}$ and $SoxR_{red}$ is somewhat shifted to the oxidized form in the presence of DNA. That is, the reduction potential of SoxR−DNA is more negative than that of DNA-free form. From the equilibrium data coupled with NADPH/CRP, we have determined that the reduction potential of DNA−SoxR complex is −320 mV versus NHE. This −320 mV reduction potential for DNA−SoxR obtained from our experiment differs markedly from that of the electrochemistry measurement (+200 mV)[17] but is more consistent with several experimental observations. First, we did not observe a reduction of the $[2Fe-2S]^{2+}$ of the DNA-bound form of SoxR by O_2^{-}.[31] O_2^{-} (with an $E_0'(O_2^{-}/O_2)$ = −160 mV[32,33]) cannot act as a reductant for $[2Fe-2S]^{2+}$ of SoxR (with an $E_0'(SoxR_{red}-DNA)/(SoxR_{ox}-DNA)$ = −320 mV). Had the previously reported value of +200 mV been applied here, the reduction would have been observed. Second, the reported reduction potentials of [2Fe−2S] proteins are in the range of −150 to −460 mV, except for Rieske proteins.[18,19] Many Rieske proteins have positive potentials (+100 to +400 mV), where histidine ligation causes upshifts of the reduction potential. Third, the dissociation constant of the $SoxR_{red}$ with the DNA (K_{dred}) can be evaluated from that of $SoxR_{ox}$ (K_{dox}) and the reduction potentials of DNA−SoxR and SoxR using eq 3.

$$E_0'(SoxR_{red}-DNA/SoxR_{ox}-DNA) = E_0'(SoxR_{red}/SoxR_{ox}) + \frac{RT}{F}\ln(K_{dox}/K_{dred}) \quad (3)$$

Using a gel mobility shift assay, Hidalgo and Demple previously determined the dissociation constant of $SoxR_{ox}$ to DNA (K_{dox}) to be 4.5×10^{-10} M.[4] From the data, K_{dred} is estimated to be 1.1×10^{-10} M, suggesting that the binding affinity of $SoxR_{red}$ with the DNA is approximately 4-fold lower than that of $SoxR_{ox}$. This is consistent with a report by Gaudu and Weiss[5] that $SoxR_{ox}$ is about twice as effective as $SoxR_{red}$ in protecting the *soxS* operator from endonucleolytic cleavage. In contrast, the electrochemistry-determined ~490 mV shift in potential would correspond to an increase in DNA binding affinity of more than 3 orders of magnitude between $SoxR_{red}$ and $SoxR_{ox}$.

The [4Fe−4S] clusters in proteins exhibit a broad range of reduction potentials (−700 to +400 mV).[18,34] The differences are generally attributed to hydrogen bonding and electrostatic effects from the surrounding proteins and solvent.[35−38] Each hydrogen-bonding interaction with the cluster can cause a potential shift of 80 mV. The substantial shift in SoxR potential upon DNA binding may reflect the structural difference between the free and DNA-bound complex. However, crystal structures of DNA-free and DNA-bound SoxR do not show large differences (Figure 6). The small shift in the SoxR

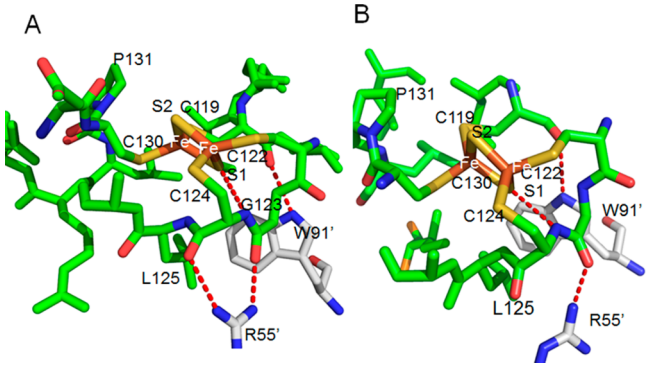


Figure 6. Structures of the [2Fe−2S] cluster of SoxR in the (A) DNA-bound and (B) DNA-free states. These structures were produced with PyMol using a structure from the Protein Data Bank (PDB ID 2ZHG and 2ZHH; ref 10).

reduction potential upon DNA binding appears to be consistent with the relatively small differences between the crystal structures of DNA-free and DNA-bound SoxR (Figure 6). For example, the hydrogen bond of the lower sulfur (S_1) of the [2Fe−2S] cluster with the amide of Gly123 is not affected upon the binding of DNA. There are some differences in the environments of the clusters between the crystal structures, for example, the [2Fe−2S] cluster is more buried in a hydrophobic core in the DNA-free form than in the DNA-bound form (Figure 7), but such differences cannot lead to the dramatic +490 mV shift indicated previously by electrochemistry.

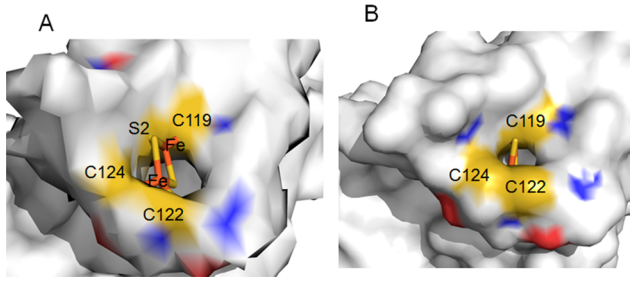


Figure 7. Surface representation of the Fe−S cluster-binding domain of DNA-bound (A) and DNA-free (B) forms. Iron and sulfur atoms of the [2Fe−2S] cluster and cysteine residues are colored.

Gorodetsky et al.[17] showed that DNA binding positively shifts the reduction potential of SoxR to 200 mV, and the shifts suggested that the DNA-bound form is primarily in the reduced form in the reducing intracellular environment. In such a case, the oxidation of SoxR may be a regulatory step and oxidative stress serves to promote oxidation of SoxR. However, the

DOI: 10.1021/bi500931w
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present data show that the reduction potential of DNA-bound SoxR (−320 mV) is slightly more negative than that of the cytoplasm (−260 to −280 mV). This suggests that SoxR would be primarily in an oxidized form in the absence of the reduction system, even without imposing oxidative stress. Under conditions of normal aerobic growth, a reductive pathway involving a NADPH-dependent specific enzyme appears to maintain SoxR in a reduced inactive form (Figure 8). Several

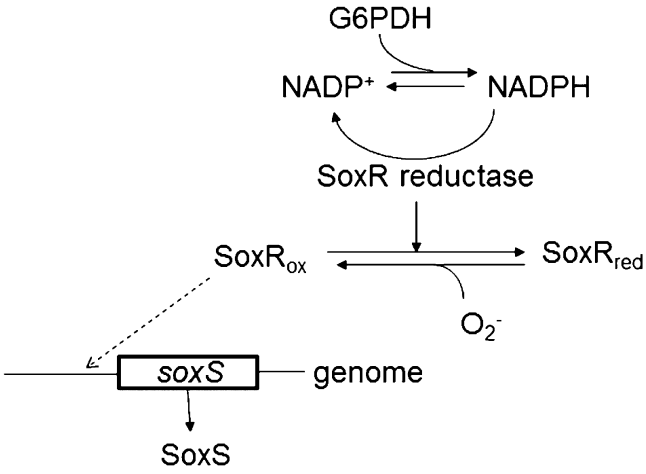

Figure 8. Schematic model for *soxRS* response of *E. coli*. Under conditions of normal aerobic growth, NADPH-dependent reductive pathway maintains the SoxR in a reduced inactive state.

studies suggested that the *SoxRS* regulon is responsive to intracellular NADPH/NADP+.[39−43] Recently, the analysis of fluorescence-activated cell sorting revealed that the transcription in SoxR is linked to the level of NADPH.[43] NADPH-dependent SoxR reduction is enzyme-mediated, allowing for a rapid adjustment to changes in cellular conditions.[44,45] The identity of SoxR reductase has not been characterized, and there still remain reducing systems other than these systems.

■ ASSOCIATED CONTENT

Supporting Information

Spectral changes of SoxR−DNA upon anaerobic addition of NADPH and CRP in the presence of indigodisulfate and phenosafranine. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors.

Funding

Research reported in this publication was supported in part by a grant-in-aid for scientific research (C) from the Ministry of Education, Science, Sports, and Culture of Japan (to K.K.)

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Prof. Emeritus Takashi Iyanagi in Himeji Institute of Technology for stimulating discussion and advise. We thank Dr. Satoshi Watanabe in Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, for stimulating discussion.

■ ABBREVIATIONS

$SoxR_{red}$, reduced ($[2Fe-2S]^{+}$) SoxR; $SoxR_{ox}$, oxidized ($[2Fe-2S]^{2+}$) SoxR; UVRR, UV resonance Raman; NADPH, nicotinamide adenine dinucleotide phosphate; CRP, NADPH−cytochrome P450 reductase; NHE, normal hydrogen electrode; PDB, Protein Data Bank; MOPS, 3-(*N*-morpholino)-propansulfonic acid; EPR, electron paramagnetic resonance; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide

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DOI: 10.1021/bi500931w
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