

## Review

# Functional Diversity of the Oxidative Stress Sensor and Transcription Factor SoxR: Mechanism of [2Fe-2S] Cluster Oxidation

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## Abstract

The [2Fe-2S] transcription activator SoxR, a member of the MerR family, functions as a bacterial stress response sensor. The response governed by SoxR is activated by the oxidation of the [2Fe-2S]. In this review, I describe functional differences between *Escherichia coli* SoxR (EcSoxR) and *Pseudomonas aeruginosa* SoxR (PaSoxR). Pulse radiolysis demonstrated that the reduced form of EcSoxR reacts directly with  $O_2^-$  with a second-order rate constant of  $5.0 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ . PaSoxR was found to undergo a similar reaction, although with a 10-fold smaller rate constant ( $4.0 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ ). This difference in rate constants may reflect distinct regulatory features of EcSoxR and PaSoxR. Specifically, mutagenesis studies have shown that Lysine residues—which are located close to [2Fe-2S] clusters, in EcSoxR, but are not conserved in PaSoxR—are essential for EcSoxR activation. In contrast, both EcSoxR and PaSoxR were found to react with various redox-active compounds (RACs), including viologens, phenazines, and quinones, with no apparent differences in the kinetic behavior or specificity of the two proteins. Importantly, both  $O_2^-$  and RACs oxidize SoxR with the same rate constants. *soxR* regulon may be induced through multiple pathways, and the activation may depend on the cellular concentration of  $O_2^-$  and RACs.

**Keywords:** iron–sulfur cluster; superoxide response; redox active compound; oxidative stress; pulse radiolysis



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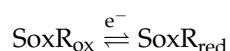
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## 1. Introduction

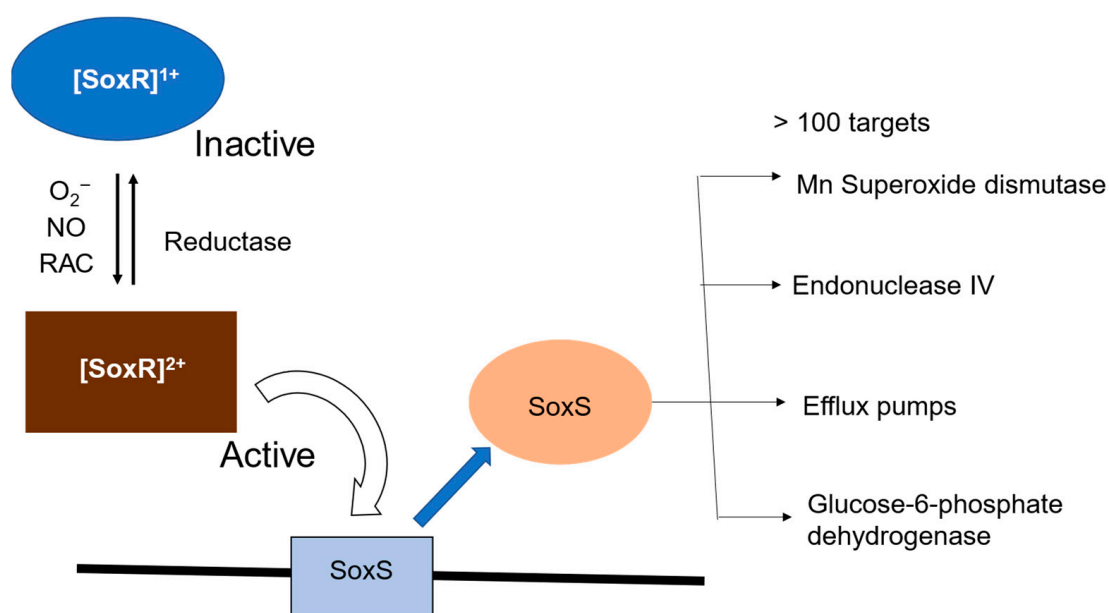
Iron–sulfur clusters are universal cofactors that play many important roles in biological processes [1–5]. Iron–sulfur clusters in signal-transducing sensor proteins are functionally distinct from most iron–sulfur clusters, which act as redox centers for electron transfer reactions. In contrast, iron–sulfur clusters in sensor proteins respond to small signaling molecules in the environment [4,6–8], resulting in structural changes such as oxidation state and cluster type. These changes may alter gene expression in cells. The SoxR, one example of such iron–cluster-containing sensor proteins, proceeds reversible oxidation and reduction between SoxR<sub>ox</sub> and SoxR<sub>red</sub>, according to the following equation.



SoxR is a 34 kDa homodimer containing one essential [2Fe-2S] cluster per polypeptide chain. Because SoxR<sub>red</sub> can be observed by EPR spectra, its redox changes can be monitored quantitatively by EPR spectroscopy. In vivo EPR spectra show that SoxR in *E. coli* (EcSoxR)

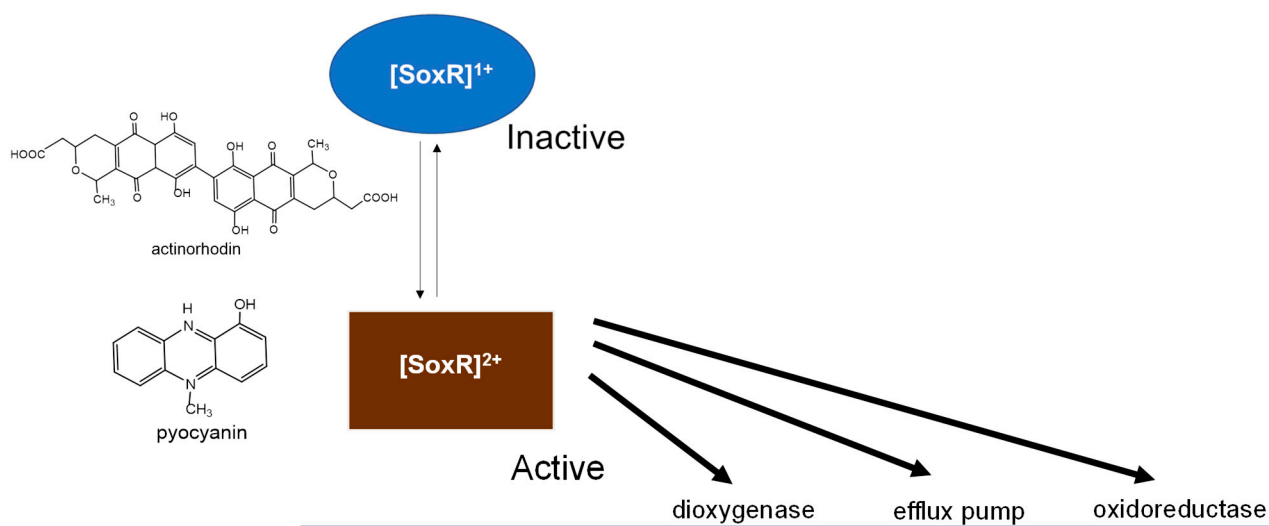
is maintained in a reduced form during normal aerobic growth [8]. When  $O_2^-$  generating compounds such as methyl viologen ( $MV^{2+}$ ) were added to *E. coli* cells, EPR signals disappeared rapidly, indicating oxidation of the EcSoxR cluster. Thus, SoxR senses oxidative stress. The resulting activated redox state is propagated to the SoxR DNA-binding domain, resulting in the activation of target gene promoters [9–13].

The target of activated SoxR is a single adjacent *soxS* gene. Increased SoxS levels induce expression of various antioxidant proteins and repair proteins [14]. This two-step *soxRS*-based regulatory mechanism controls the expression of genes encoding antioxidant defense proteins such as Mn-containing superoxide dismutase (SOD), glucose-6-phosphate dehydrogenase, endonuclease IV, and outer membrane drug efflux pump, as shown in Figure 1.



**Figure 1.** The mechanism underlying transcription of *soxRS* regulon in *E. coli*. SoxR is activated by exposure to  $O_2^-$ -generating compounds, redox active compounds (RACs), or nitric oxide (NO). The target of activated SoxR is the *soxS* gene. Increased SoxS induces expression of various antioxidant proteins.

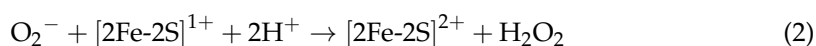
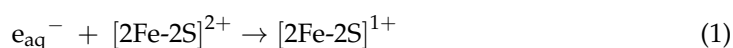
A number of SoxR homologs have been identified in various bacteria [15], all of which contain a SoxR-specific cysteine motif and DNA binding sites. In contrast to those in *E. coli* and related enteric bacteria, the majority of SoxR regulons in non-enteric bacteria such as *P. aeruginosa* and *Streptomyces coelicolor* lack genes involved in oxidative stress and detoxification [15]. Some of these bacteria produce redox-active compounds (RACs) independent of oxidative stress [15,16]. PaSoxR is activated by endogenous RAC antibiotic compounds, such as pyocyanin (Pyo) and actinorhodin (Act) [15–18], which are the physiological signals for the regulation of quorum-sensing-regulated genes. These SoxR target genes regulate genes involved in export (via the *mexGHI-opmD* RND efflux pump [19]) and the metabolism of small molecules [19], including RAC antibiotics and endogenous pigments (Figure 2). In these species, SoxR appears to function as a defense against the toxicity of RACs.



**Figure 2.** SoxR response in non-enteric bacteria.

## 2. Direct Oxidation of [2Fe-2S] Cluster in SoxR by O<sub>2</sub>

An important question relates to the chemical nature of the signal sensed by SoxR. The first proposal SoxR-activating signal identified was O<sub>2</sub><sup>−</sup> [20], but the mechanism by which O<sub>2</sub><sup>−</sup> activates SoxR remains unclear [21]. Gu and Imaly [22] reported that EcSoxR is not directly activated by O<sub>2</sub><sup>−</sup>, but RACs. To investigate the oxidation of EcSoxR by O<sub>2</sub><sup>−</sup>, I followed the reactions that occurred following pulse radiolysis of oxygen-saturated aqueous solutions in the presence of EcSoxR<sub>ox</sub>, which were previously determined [13]. Under these experimental conditions, most of the hydrated electrons (e<sub>aq</sub><sup>−</sup>) are converted to O<sub>2</sub><sup>−</sup>, while a part of the e<sub>aq</sub><sup>−</sup> population rapidly reduced the [2Fe-2S] cluster. The initial rapid absorption changes due to reduction in the [2Fe-2S] cluster partially recovered on a millisecond time scale. It is noted that the recovery was inhibited by the addition of Cu/Zn-SOD. From pulse radiolysis results, the reaction schemes can be summarized by reactions (1) and (2):

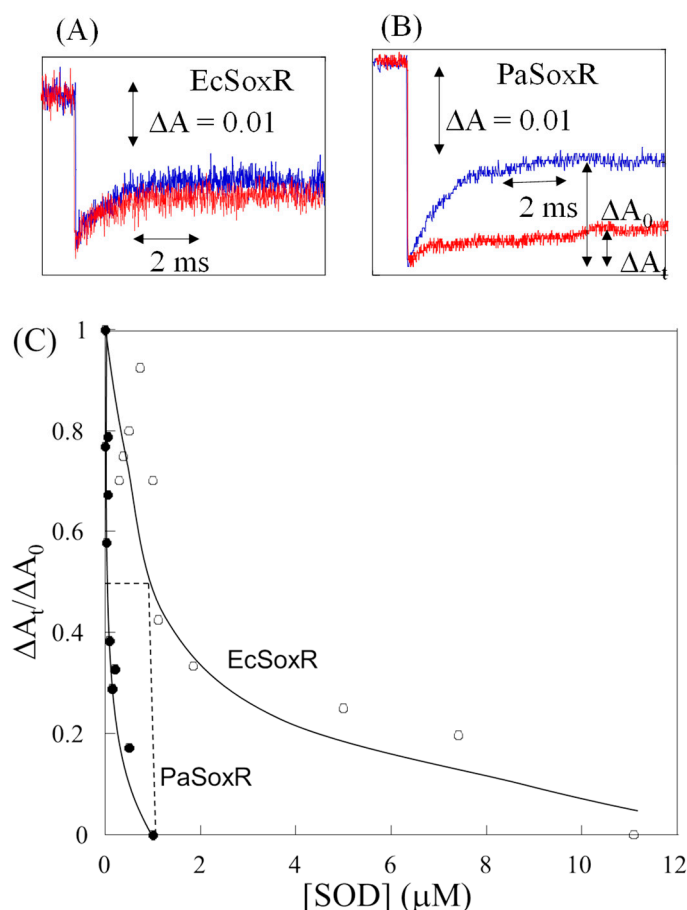


where [2Fe-2S]<sup>2+</sup> and [2Fe-2S]<sup>1+</sup> are oxidized and reduced forms of SoxR, respectively.

Reaction (2), observed in SoxR, represents the first reported example of O<sub>2</sub><sup>−</sup> oxidation, a reduced form of iron–sulfur protein. Whether other [2Fe-2S] proteins show similar reactivity was tested by examining their reactions with O<sub>2</sub><sup>−</sup> using spinach ferredoxin and adrenodoxin as examples. However, unlike the case of SoxR, after rapid reduction in these [2Fe-2S] proteins by e<sub>aq</sub><sup>−</sup>, the recovery of absorption changes was not observed. On the other hand, in the reaction of the oxidized form of iron–sulfur proteins, reactions between iron–sulfur proteins and O<sub>2</sub><sup>−</sup> with second-order rate constants ranging from 10<sup>6</sup> to 10<sup>7</sup> M<sup>−1</sup> s<sup>−1</sup> [23–25] were reported. In these cases, the reactions were irreversible and were accompanied by the degradation of the iron–sulfur cluster. By contrast, reaction (2) in SoxR is reversible. Therefore, it can be said that reaction (2) of SoxR is a specific characteristic of SoxR protein.

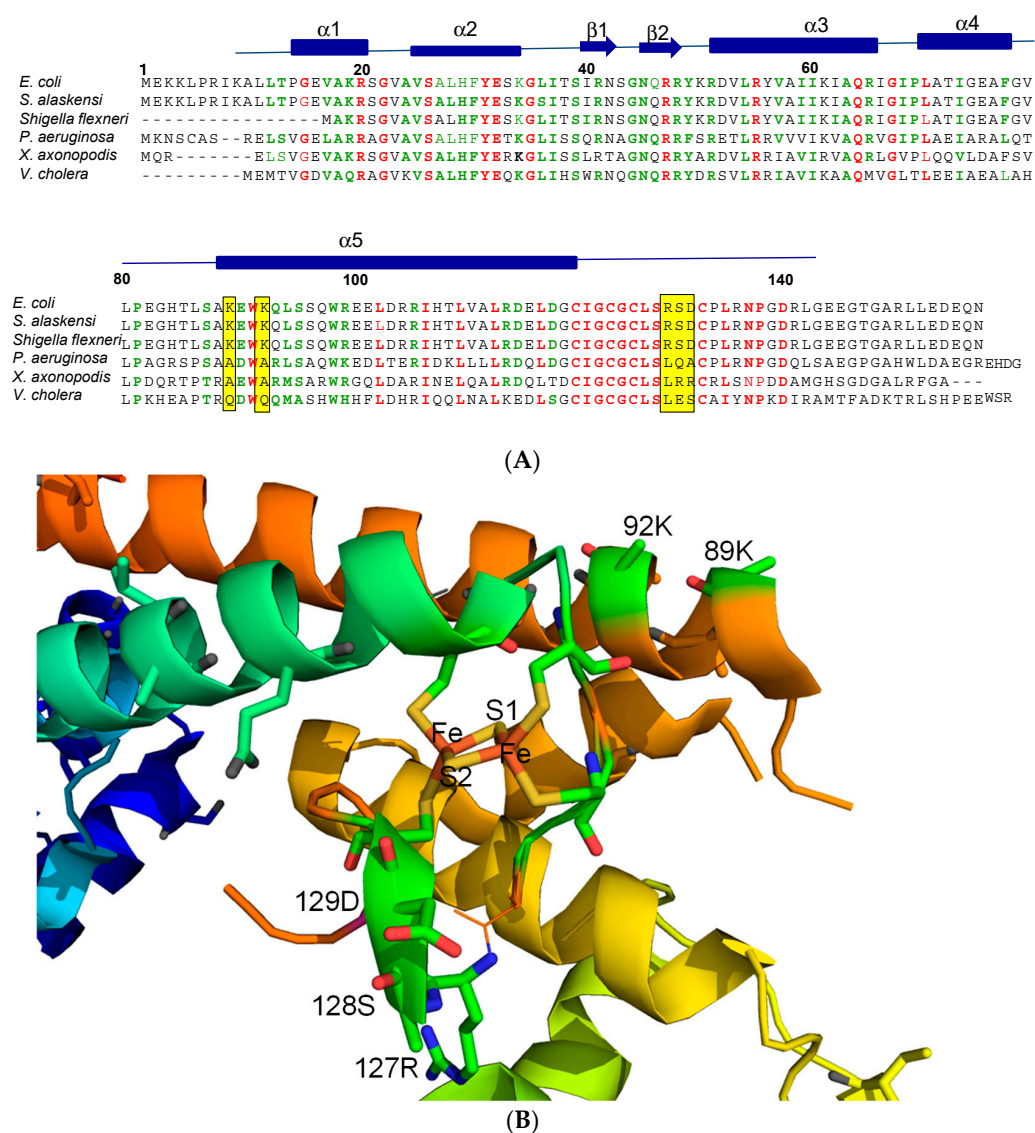
Pulse radiolysis revealed that a similar process was at work in PaSoxR. To compare the reactions of EcSoxR and PaSoxR, we studied the inhibition by SOD oxidation of SoxR<sub>red</sub> (Figure 3). The oxidation of PaSoxR was completely inhibited by 0.5 μM SOD (Figure 3B), whereas the same concentration of SOD inhibited EcSoxR by only 20% (Figure 3A). This suggests that the reactivity of O<sub>2</sub><sup>−</sup> toward EcSoxR is much greater than toward PaSoxR.

The SOD dependence of absorption changes at 420 nm for EcSoxR is presented in Figure 3C. Based on the concentration of SOD with the half-maximal inhibition of SoxR<sub>red</sub> and the rate constant for the reaction of O<sub>2</sub><sup>•−</sup> with SOD, we obtained a second-order rate constant of  $5.0 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$  for the reaction of O<sub>2</sub><sup>•−</sup> with EcSoxR<sub>red</sub>. In contrast, the rate constant for PaSoxR<sub>red</sub> with O<sub>2</sub><sup>•−</sup> was calculated to be  $4.0 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ . The difference in rate constant reflects the distinct regulatory roles for EcSoxR and PaSoxR in response to their physiological activation by O<sub>2</sub><sup>•−</sup>. The mechanism underlying the different sensitivities of various SoxRs to O<sub>2</sub><sup>•−</sup> remains an important question.



**Figure 3.** SOD effects on the oxidation of SoxR. Changes in absorbance measured after pulse radiolysis of EcSoxR (A) and PaSoxR (B) in the absence (blue line) or presence (red line) of 0.5 μM SOD. (C) The absorbance changes of (○) EcSoxR and (●) PaSoxR at time (ΔA<sub>t</sub>), normalized to the total absorbance change (ΔA<sub>0</sub>) plotted against the concentration of SOD, Reprinted from [13].

These data clearly suggest that slight alteration in structure between highly homologous SoxR proteins can lead to functional differences. Figure 4A compares sequence alignments for SoxR proteins from different enteric and non-enteric bacteria. SoxR homologs from *E. coli*, an enteric species, contain two lysine residues, which are substituted with alanine residues in the SoxR of most non-enteric species [26]. In the crystal structure of EcSoxR [27], Lys89 and Lys92, located immediately upstream of dimerization helix 5, are close to the [2Fe-2S] cluster (Figure 4B). In addition, SoxRs from most species of enteric bacteria contain a conserved three-residue hydrophilic motif (R127S128D129) in the proximity of [2Fe-2S] clusters that affect SoxR sensitivity to RACs [26] and it is not conserved in SoxR from other non-enteric bacteria [26].



**Figure 4.** (A) Sequence alignments of SoxR proteins from enterics *E. coli*, *Sphingopyxis alaskensis*, and *Shigella flexneri* and non-enteric (*P. aeruginosa*, *Xanthomonas axonopodi*, *Vibrio cholerae*) bacteria. Identical and conserved residues among SoxR proteins are denoted in red and green, respectively. Hypervariable residues and the hypervariable motif are highlighted in yellow. (B) Structure of EcSoxR in the region near the [2Fe-2S] cluster. The structure was produced with PyMol using a structure from the Protein Data Bank (entry 2ZHG). Reproduced from [26]. Copyright 2016 American Chemical Society.

Mutagenesis experiments have investigated the sensitivity to  $O_2^-$ , focusing on several amino acids that are not conserved among SoxR homologues. Specifically, the EcSoxR mutants (K89A, K92A, K89AK92A, D129A, and R127LS128QD129A) and PaSoxR mutants (A87K, A90K, and L125RQ126SA127D) were prepared, and their  $O_2^-$  sensitivity was determined by examining the effects of concentration on SOD inhibition [26]. Table 1 shows the rate constants for EcSoxR and PaSoxR mutants. Although the rate constants for the K89A ( $3.8 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ ) and K92A ( $2.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ ) mutants were only slightly affected by the indicated substitutions, the rate constant for the double-Lys K89AK92A mutant ( $3.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ ) was dramatically affected. The rate constant of  $3.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$  was 10 times smaller than that of wild-type SoxR. Conversely, the corresponding substitution of K to A in PaSoxR at residues (A87K) or (A90K) increased rate constants from approximately 10-fold to  $2.1 \times 10^8$  and  $5.4 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ , respectively (Table 1). Therefore, substituting

an alanine residue for a lysine residue in the [2Fe-2S] region of EcSoxR is sufficient to transform the  $O_2^-$ -sensitivity of EcSoxR such that it mimics that of PaSoxR. The effects of the electrostatic charge of amino acid residues were further analyzed by replacing both positive charged both Lys89 and Lys92 residues in EcSoxR with negatively charged glutamate (K89EK92E). The resulting charge reversal from positive to negative decreased the rate constant; in contrast, substituting arginine, another positively charged amino acid, for these Lysine (K89RK92R) did not change rate constants (Table 1). Thus, these Lysine residues are responsible for the high diffusion-limited rate constant. Collectively, these results demonstrate that these electrostatically positive Lysine residues are critical for the reaction of  $O_2^-$  with the [2Fe-2S] cluster in EcSoxR and suggest that they guide  $O_2^-$  into the active site on the surface of SoxR. These findings are consistent with the previously reported involvement of Lysine residues in enzymatic reactions with  $O_2^-$  [28–30].

**Table 1.** Second-order rate constants in  $O_2^-$  reactions with the reduced forms of wild-type and mutant SoxR proteins.

	$k \times 10^8 \text{ (M}^{-1} \text{ s}^{-1}\text{)}$
<i>E. coli</i>	
WT	5.0
R127LS128QD129A	4.8
D129A	5.0
K89A	3.8
K92A	2.2
K89AK92A	0.33
K89RK92R	4.7
K89EK92E	0.31
<i>P. aeruginosa</i>	
WT	0.4
A87K	2.1
A90K	5.4
L125RQ126SA127D	0.4

SoxR from most species of enteric bacteria contains a three-residue hydrophilic motif (R127S128D129) in the vicinity of [2Fe-2S] clusters. These residues are not conserved in the SoxR of other bacteria. It has been proposed that the presence of this conserved motif affects the sensitivity of SoxR to RACs [18,31] and is responsible for the observed species-specific differences in RAC sensitivity. However, unlike the above Lys substitutions, the RSD→LQA substitution in EcSoxR and LQA→RSD substitution in PaSoxR did not affect  $O_2^-$  reaction rates (Table 1).

### 3. Response of SoxR [2Fe-2S] to RACs

It has been shown that EcSoxR responds to endogenous, as well as various synthetic RACs [17,18,31,32], suggesting that EcSoxR<sub>red</sub> is oxidized directly, but nonspecifically, by RACs. In contrast, PaSoxR is only activated by endogenous molecule RACs and appears to respond to more restricted molecules. The sensitivity and specificity of various RACs were examined by investigating the kinetics of the oxidation of the [2Fe-2S] cluster in SoxR using pulse radiolysis [33]. Pulse radiolysis enables reduction of  $[2Fe-2S]^{2+}$  in SoxR<sub>ox</sub> with a hydrated electron ( $e_{aq}^-$ ) within 1  $\mu$ s (reaction 1), which permits subsequent oxidation of  $[2Fe-2S]_{red}$  by RACs such as MV<sup>2+</sup> (reaction 3).

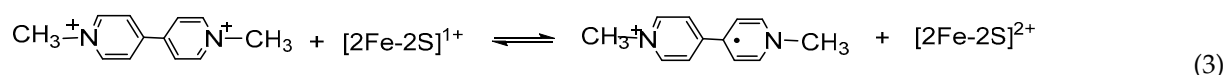
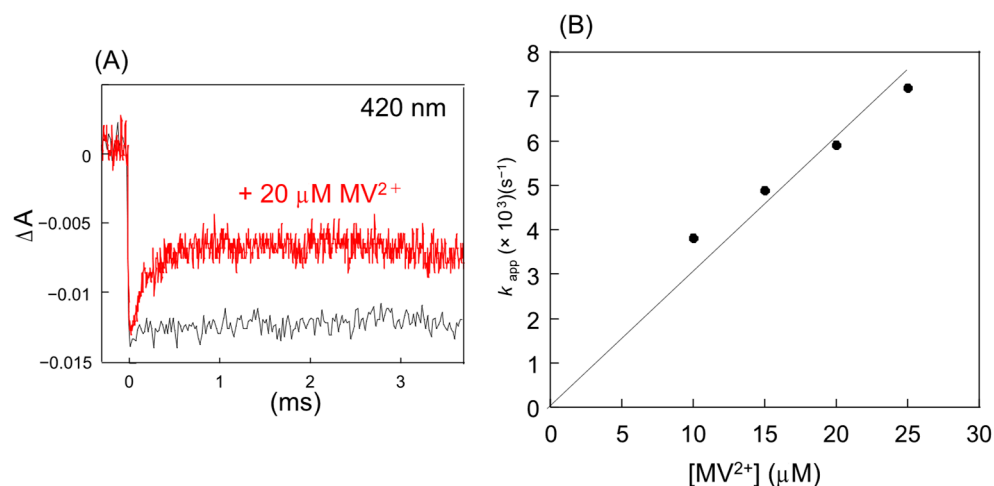


Figure 5 shows the absorption changes during pulse radiolysis of PaSoxR in the absence and presence of  $MV^{2+}$ . After the initial decreasing absorbance, a subsequent partially reverse process was observed in the presence of  $MV^{2+}$  on a millisecond time scale. From these findings, it can be inferred that the  $[2Fe-2S]$  cluster of SoxR<sub>ox</sub> is initially reduced by  $e_{aq}^-$ , and subsequently becomes partially reoxidized, implying that the recovery process reflects the oxidation of SoxR<sub>red</sub> by  $MV^{2+}$  (reaction 3). Similar absorption changes were observed in EcSoxR.



**Figure 5.** (A) Changes in absorbance at 420 nm after pulse radiolysis of PaSoxR. Samples contained 70  $\mu M$  PaSoxR, 20 mM potassium phosphate buffer (pH 7.0), 10 mM potassium/sodium tartrate, 0.5 M KCl, and 0.1 M t-butanol, to which 20  $\mu M$   $MV^{2+}$  was further added in red colored. (B) Concentration dependence of  $MV^{2+}$  versus  $k_{app}$  in PaSoxR.

To determine the second-order rate constants in the reaction of  $MV^{2+}$  with PaSoxR<sub>red</sub>, we used conditions in which the concentrations of the RAC (10–25  $\mu M$ ) was much higher than that of SoxR<sub>red</sub> (0.5–1  $\mu M$ ). Under these conditions, the oxidation of SoxR<sub>red</sub> obeyed pseudo-first-order kinetics, with the observed rate constant increasing linearly with RAC concentration. The rate constant of the reaction, obtained from the slope in Figure 5B was determined to be  $3.0 \times 10^8 M^{-1}s^{-1}$ . Similar absorption changes were observed in the presence of other RACs, including pyocyanin (Pyo), phenazine methosulfate (PMS), duroquinone (Dur), and diquat (Dq). The second-order rate constants increased in the order  $Dur < MV^{2+} < Dq < Pyo < PMS$  (Table 2) and were found to correlate with the electrode potential of the RAC. In all cases, a rapid decrease phase was followed by a slower recovery phase. Notably, absorbance changes during the slower reoxidation phase varied depending on the specific RACs. The incomplete reoxidation of the  $[2Fe-2S]$  cluster may be explained by a rapid equilibrium for electron transfer between the RAC and the  $[2Fe-2S]$  cluster (reaction 3).

**Table 2.** Rate constants of  $[2Fe-2S]$  cluster of EcSoxR, and PaSoxR, with RACs and the electrode potentials  $E_0$  (vs NHE) at pH 7.0.

RACs	$E_0$ (vs. NHE) (mv)	EcSoxR $k (\times 10^8 M^{-1}s^{-1})$	PaSoxR $k (\times 10^8 M^{-1}s^{-1})$
$MV^{2+}$	−440 [34]	3.0	3.0
Dq	−358 [35]	5.7	5.5
Dur	−260 [35]	2.1	2.6
Pyo	−34 [36]	7.1	6.8
PMS	+80 [36]	16.0	14.0

The pulse radiolysis results presented here show that the  $[2\text{Fe-2S}]_{\text{red}}$  cluster of EcSoxR and PaSoxR react with RACs with electrode potentials in the range of  $-440\text{ mV}$  ( $\text{MV}^{2+}$ ) [34] to  $+80\text{ mV}$  (PMS) [36]. Importantly, these results are consistent with the responsiveness of SoxR to RACs observed in vivo [18,22,32]. In comparison of the sensitivity profiles of SoxR towards RACs, Singh et al. showed that both EcSoxR and PaSoxR responded to all RACs, except Act [18].

SoxR homologs exhibit different selectivity toward RACs with each displaying distinctive effective concentrations and cellular response times [31]. However, our study found that these differences are not reflected in the kinetics of the reactions. Specifically, pulse radiolysis revealed no difference in the reactivity of the  $[2\text{Fe-2S}]$  cluster between EcSoxR and PaSoxR, despite their differing cellular response times to  $\text{MV}^{2+}$  [22].

#### 4. Physiological Significance of SoxR Response to $\text{O}_2^-$ and RACs

In this review, from the in vitro data,  $\text{O}_2^-$  directly oxidizes purified SoxR [11,13,26], suggesting that  $\text{O}_2^-$  itself is the signal that activates SoxR. However, it is not known whether  $\text{O}_2^-$  can interact with SoxR under high levels of SOD and a low level of SoxR constitutively under normal cellular conditions [25,37]. In addition, the following in vivo experiments have shown that  $\text{O}_2^-$  is not the signal for SoxR. Firstly, endogenous  $\text{O}_2^-$  in SOD- mutants does not activate SoxR in SOD-deficient cells [22,38]. Secondly RACs such as  $\text{MV}^{2+}$  can activate SoxR in anoxic conditions, in the presence of alternative respiratory acceptors [22]. The *soxR* regulon may be induced through multiple pathways. Since both  $\text{O}_2^-$  and RACs oxidize SoxR with the same rate constants, the activation depends on the cellular concentration of  $\text{O}_2^-$  and RACs.

The data presented here provide new insight into electron transfer between RACs and the  $[2\text{Fe-2S}]$  cluster of SoxR homologs. The observed oxidation of the  $[2\text{Fe-2S}]$  in SoxR proceeds via direct electron transfer from the  $[2\text{Fe-2S}]$  cluster to the RAC. It is likely that structurally distinct RACs can gain access through the solvent-exposed surface area allowing rapid electron transfer between the  $[2\text{Fe-2S}]$  cluster and RACs. An X-ray analysis of DNA-free EcSoxR [24] showed that the upper sulfur atom (S2) and one of the Fe atoms are completely exposed to the solvent. A similar mechanism may take place at the SoxR active site in PaSoxR. The oxidizability of SoxR<sub>red</sub> is assisted by the low potential of its  $[2\text{Fe-2S}]$  cluster in EcSoxR ( $-285\text{ mV}$ ) [39] and PaSoxR ( $-290\text{ mV}$ ) [39], which provide a strong driving force for electron transfer to even the moderate oxidant ( $-440\text{ mV}$ ),  $\text{MV}^{2+}$  [33].

#### 5. Conclusions and Future Perspectives

In this review, I followed reactions of the  $[2\text{Fe-2S}]$  cluster of SoxR with  $\text{O}_2^-$  and RACs directly. Both rate constants were almost a diffusion-controlled rate and characteristic of the individual SoxRs. The next major goal now is to investigate the factors that control the iron-sulfur cluster. To understand how iron-sulfur clusters respond to the molecules in the environment, future progress at the boundary of physical chemistry, biological chemistry, and inorganic chemistry will be crucial.

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