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Redox Sensing by Prokaryotic Transcription Factors

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ABSTRACT. Prokaryotic cells employ redox-sensing transcription factors to detect elevated levels of reactive oxygen species and regulate expression of antioxidant genes. In *Escherichia coli*, two such transcription factors, OxyR and SoxR, have been well characterized. The OxyR protein contains a thiol–disulfide redox switch to sense hydrogen peroxide. The SoxR protein uses a 2Fe–2S cluster to sense superoxide generated by redox-cycling agents, as well as to sense nitric oxide. Both proteins are turned on and off with very fast kinetics (~ minutes), allowing rapid cellular responses to oxidative stress. The mechanisms by which these and other prokaryotic proteins sense redox signals have provided useful paradigms for understanding redox signal transduction in eukaryotic cells. BIOCHEM PHARMACOL **59**;1:1–6, 2000. © 1999 Elsevier Science Inc.

KEY WORDS. OxyR; SoxR; disulfide bond; iron–sulfur cluster; oxidative stress

In recent years, redox regulation, defined as the modulation of protein activity by oxidation and reduction, has become recognized as an important mechanism for controlling cellular activities [1]. The crucial first step in redox regulation is the perception of redox-signaling molecules such as superoxide, nitric oxide, and hydrogen peroxide. The understanding of redox regulation thus hinges on the identification and characterization of proteins that sense reactive oxygen and nitrogen species. In prokaryotic cells, redox-sensing transcription factors are employed to sense elevated levels of superoxide and hydrogen peroxide and activate the expression of antioxidant genes. The simple regulatory circuitry of prokaryotic cells and well-developed genetic tools for these systems have greatly facilitated the elucidation of the chemistry of redox sensing by these proteins. This review will focus on the two best-characterized redox-sensing transcription factors, OxyR and SoxR, which sense hydrogen peroxide and superoxide, respectively. It is our belief that the principles learned from studying these prokaryotic regulators will directly apply to eukaryotic redox-sensing proteins.

OxyR—SENSING HYDROGEN PEROXIDE BY A THIOL–DISULFIDE SWITCH

The *E. coli* OxyR transcription factor activates the expression of several antioxidant defensive genes in response to elevated levels of hydrogen peroxide [2, 3], including *kat*G (hydroperoxidase), *ahpCF* (alkyl hydroperoxide reductase), *oxyS* (a regulatory RNA), *dps* (a non-specific DNA-binding protein), *fur* (ferric uptake regulation), *gor*A (glutathione reductase), and *grx*A (glutaredoxin). An *E. coli oxyR2* constitutive mutant overexpresses the OxyR-regulated genes and is more resistant to hydrogen peroxide than its wild-type parent strain [4]. In contrast, *oxyR*-deletion mutants are hypersensitive to hydrogen peroxide and fail to activate the expression of OxyR-regulated genes in response to hydrogen peroxide. The 34 kDa OxyR protein shares homology with the LysR family of bacterial regulators. Like other members of the LysR family, OxyR possesses a helix-turn-helix DNA-binding motif (HTH) in its N-terminal domain. OxyR forms a tetramer in solution. Transcriptional activation of antioxidant genes by OxyR is thought to occur via direct contact with RNA polymerase. Early studies demonstrated that OxyR from *E. coli* exists in oxidized and reduced forms, but that only the former activates transcription [5]. Recent studies showed that oxidation by peroxide leads to the formation of a disulfide bond in OxyR that triggers the activation of the transcription factor [6]. Mutation of either of the two conserved cysteines (C199 and C208) in OxyR to serine or alanine abolished the ability of the transcription factor to sense hydrogen peroxide, both *in vivo* and *in vitro*. Mass spectrometry measurements and thiol–disulfide quantitation assays on the purified protein showed that C199 and C208 are in disulfide bond form in the oxidized OxyR, and in dithiol form in the reduced OxyR. These findings suggest that OxyR activation and deactivation is a consequence of the C199–C208 disulfide bond formation and reduction (Scheme 1).

A direct demonstration of OxyR disulfide bond formation *in vivo* was achieved by exploiting thiol alkylation chemistry [7]. Thiols but not disulfide bonds can be alkylated by iodoacetamide derivatives. Therefore, alkyla-

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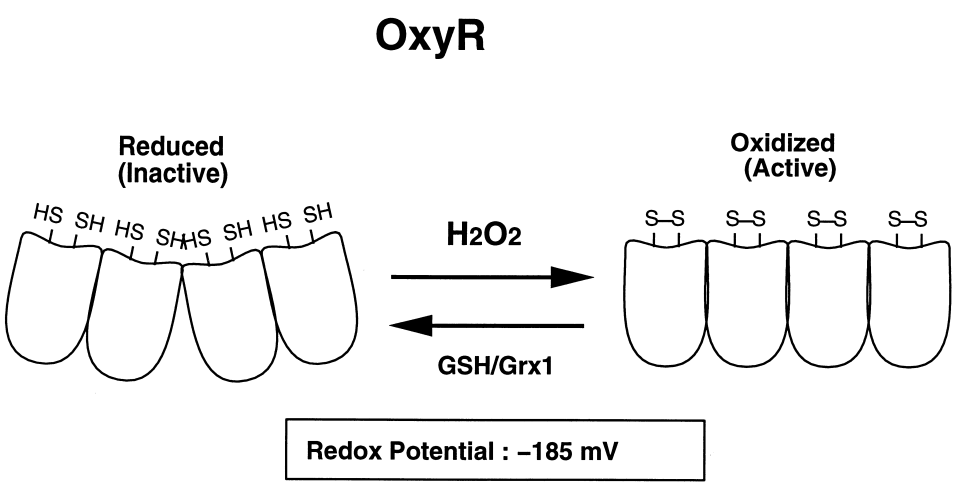

SCHEME 1. Model for OxyR activation and deactivation.

tion of OxyR proteins with AMS* adds ~1 kDa mass to the reduced but not oxidized OxyR, allowing separation of the two forms of the OxyR protein by size on polyacrylamide gels. This approach was applied to examine the *in vivo* kinetics of OxyR oxidation and reduction. The OxyR protein was completely oxidized by hydrogen peroxide within 30 sec in logarithmically growing wild-type cells, and remained oxidized for about 5 min. The *in vitro* kinetics was similar: oxidation of the OxyR protein by hydrogen peroxide was complete within 30 sec and maintained for a period of 10–30 min. The OxyR protein oxidation and reduction kinetics revealed by the AMS-alkylation method paralleled the increase and decrease of RNAs transcribed from OxyR-regulated genes.

The kinetic study clearly indicates that OxyR remains oxidized and activated for only a finite period of time. What factors are responsible for the reduction and deactivation of the OxyR disulfide bond? Two major disulfide-reduction systems are present in the *E. coli* cytosol: glutathione together with the glutaredoxin proteins and thioredoxin reductase together with thioredoxin [8, 9]. *In vivo*, the OxyR-regulated response was found to be prolonged in mutants lacking GSH or glutaredoxin 1, but not in mutants lacking thioredoxin or thioredoxin reductase. *In vitro* transcription assays showed that glutaredoxin 1 catalyzes the reduction of OxyR by GSH. Therefore, both *in vivo* and *in vitro* results indicate that oxidized OxyR is reduced by glutaredoxin 1 in the cell. Interestingly, since the gene encoding glutaredoxin 1 is itself regulated by OxyR, the OxyR response is autoregulated. The redox potential of OxyR was determined to be −185 mV, a value that ensures that OxyR is in the dithiol form in the reducing environment of the cell (thiol–disulfide redox potential −280 mV), and thus is poised to sense hydrogen peroxide [6].

The AMS-alkylation experiments also showed that OxyR protein is extremely sensitive to hydrogen peroxide. The minimum hydrogen peroxide concentration required to completely oxidize OxyR *in vivo* is 5 μM, and the minimum concentration required *in vitro* is between 0.05 and 0.2 μM [7]. These concentrations are consistent with the submicromolar levels required for the OxyR-dependent activation of a *katG-lacZ* fusion *in vivo* [10]. The findings suggest that the two active-site cysteines (C199 and C208) of the OxyR protein have evolved to sense levels of hydrogen peroxide that just exceed the normal tolerable intracellular concentrations. Given the fact that there are millimolar levels of GSH inside the cell compared to submicromolar levels of OxyR, the reaction between OxyR and hydrogen peroxide must be highly specific. An important direction for future studies will be to elucidate how the OxyR protein structure ensures such high reaction specificity. Thiol oxidation to disulfide bond by hydrogen peroxide is generally believed to proceed through the formation of sulfenic acid (−SOH) intermediate, followed by nucleophilic attack by the second thiol group [11–13]. It will be interesting to determine how the conserved residues surrounding C199 and C208 (Fig. 1), including H198 and R201, influence the reactivity of the OxyR protein.

SoxR—SENSING SUPEROXIDE BY AN IRON–SULFUR CLUSTER

The *E. coli* SoxR transcription factor activates the expression of a single gene *soxS* in response to exposure to superoxide-generating agents and to nitric oxide. The elevated levels of the SoxS protein (encoded by *soxS*) then lead to increased expression of several genes, including *sodA* (superoxide dismutase), *zwf* (glucose-6-phosphate dehydrogenase), *fpr* (NADPH:flavodoxin oxidoreductase), *fldA* (flavodoxin 1), *fumC* (fumarase C), *acnA* (aconitase), *nfo* (endonuclease IV), and *micF* (a regulatory RNA). Activities such as superoxide dismutase (superoxide scavenging) and endonuclease IV (DNA repair) have obvious roles in the antioxidant defense response. In addition to protecting against oxidative damage, the SoxRS regulon

* *Abbreviations:* AMS, 4-acetamido-4′-maleimidylstilbene-2-2′-disulfonic acid; PerR, peroxide regulon repressor; and EPR, electron paramagnetic resonance.

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                                                C199        C208
E.coliOxyR EDHPWANRECVPMADLAGEKLLMLEDGHCLRDQAMGFCFEAGAD-EDT
E.caroOxyR QDHPWANRERVAMSDLAGEKLLMLEDGHCLRDQAMGFCFQAGAD-EDT
X.campOxyR EGHPLSRHDSMTLDDLSEQRLLLLEDGHCLRDQALDVCHLAGAL-EKS
S.viriOxyR LEHGLGGREGIPRKALRELNLLLLDEGHCLRDQALDICREAGSAGVAA
B.aborOxyR TNDHTVLASPMTQNHAALERLLLEEGHCMRDQALAVCTLPSQR-QLV
M.mariOxyR PGHPMADRHGVPVAALSELPLLLLDEGHCLRDQALDVCQNAGVRAELA
A.calcOxyR K-HDKHSVNAHSLDDLDLSRLMLLEEGHCLRDHALSACPIGERK-NDN
H.inflOxyR EHHPWAQESKLPMNQLNGQEMLMLDDGHCLRNQALDYCFTAGAK-ENS
Y.pesiOxyR ADHPWANRERVEMHELAGEKLLMLEDGHCLRDQAMGFCFQAGAD-EDT
A.actiOxyR EQHPWANENSVSMSLLKDCEILMLDDGHCLRNQALGYCFTAGAR-ENA
V.cholOxyR CDHAWAARDEVDMLELKGKTVLALGDGHCLRDQALGFCFAAGAK-DDE
P.multOxyR ENHPWANERTIAMNRLNGCEMLMLDDGHCLRDQTIGYCFSAGAK-ENA
B.pertOxyR HDHEWAQRKAIDAQDLKQQTMLLLGSGHCFRDQVLEVCPELSRFSASS
P.aeruOxyR ADHPWTAKASIDSELLNDKSLLLLGEGHCFRDQVLEACPTVRKGDENK
N.gon OxyR KGHSFEELDAVSPRMLGEEQVLLLTEGNCMRDQVLSSCSELAAKQRIQ
P.gingOxyR RCEPLFEQDVIRTTEVNPHRLWLLDEGHCFRDQLVRFCQMKGLHERQT
Consensus                          ^  * ^*^* * ^ ^  *
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FIG. 1. Active-site sequence comparison of OxyR homologues from *Escherichia coli* (E.coliOxyR), Erwinia carotovora (E.caroOxyR), *Xanthomonas campestris* (X.campOxyR), *Steptomyces viridosporus* (S.viriOxyR), *Brucella abortus* (B.aborOxyR), *Mycobacterium marinum* (M.mariOxyR), *Acinetobacter calcoaceticus* (A.calcOxyR), *Haemophilus influenzae Rd* (H.inflOxyR), *Yersinia pestis* (Y.pesiOxyR), *Actinobacillus actinomycetemcomitans* (A.actiOxyR), *Vibrio cholerae* (V.cholOxyR), *Pasteurella multocida* (P.multOxyR), *Bordetella pertussis* (B.pertOxyR), *Pseudomonas aeruginosa* (P.aeruOxyR), *Neisseria gonorrhoeae* (N.gonOxyR), and *Porphyromonas gingivalis* (P.gingOxyR). The "*" signifies absolute conservation, and the "^" signifies a high degree of conservation. The two conserved cysteines have been shown to form a disulfide bond upon hydrogen peroxide oxidation.

confers resistance to antibiotics, to organic solvents, and to heavy metals. Activation of the SoxRS regulon is also important for *E. coli* resistance to nitric oxide-generating macrophages [2]. SoxR is a 17 kDa polypeptide with homology to the mercury-dependent MerR regulator of *E. coli*. It contains a helix-turn-helix (HTH) DNA-binding motif in the N-terminal domain. SoxR forms a homodimer in solution, and each a dimer contains two redox-active [2Fe–2S] cluster [14, 15]. Site-directed mutagenesis studies have shown that the four conserved cysteine residues clustered in the C-terminal domain of the SoxR polypeptide provide the ligands for the [2Fe–2S] clusters [16] (Fig. 2). The [2Fe–2S] clusters are essential for the transcriptional activity of SoxR *in vitro* and *in vivo*. *In vitro* transcription experiments showed that SoxR becomes activated to stimulate the transcription of its target gene *soxS*

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E.coliSoxR      IHTLVALRDELDGCIGCGCLSRSDCPLRNPGD
S.typhSoxR      IHTLVALRDELDGCIGCGCLSRSDCPLRNPGD
P.aeruSoxR      IDKLLLLRDQLDGCIGCGCLSLQACPLRNPGD
S.violSoxR      IEQLLALRDGLEDCIGCGCLSVRDCPLTNPYD
C.violSoxR      IEALCRLRDQLDSCIGCGCLSLERCKLYNPDD
B.pertSoxR      ILXLTQLRDQLDGCIGCGCLSLRECPLRNPDD
V.cholSoxR      IQQLNALKEDLSGCIGCGCLSLESCAIYNPKD
Consensus       *  *  *^^ *  ***********  * ^ ** *
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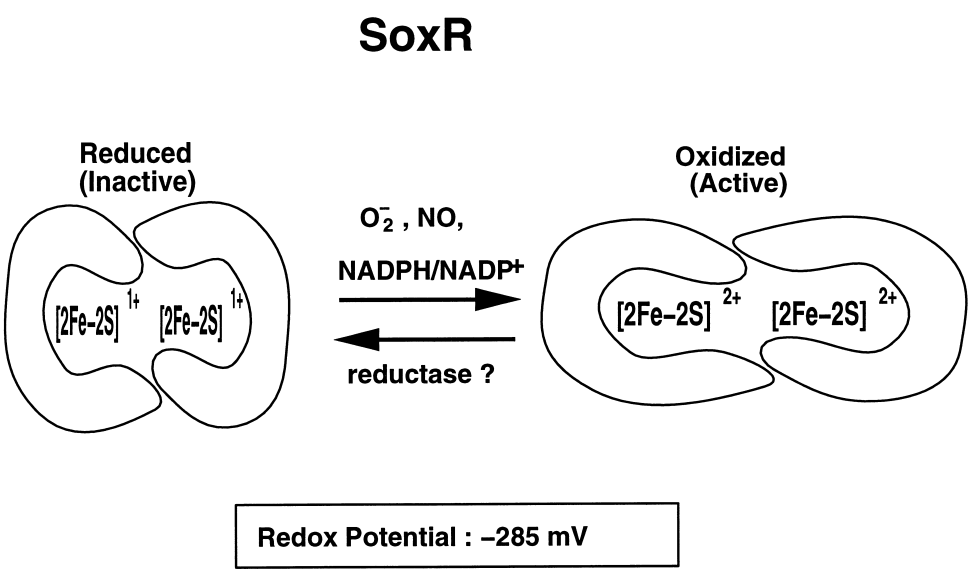
FIG. 2. Active-site sequence comparison of SoxR homologues from *Escherichia coli* (E.coliSoxR), *Salmonella typhimurium* (S.typhSoxR), *Pseudomonas aeruginosa* (P.aeruSoxR), *Streptomyces violaceoruber* (S.violSoxR), *Chromobacterium violaceum* (C.violSoxR), *Bordetella pertussis* (B.pertSoxR), and *Vibrio cholerae* (V.cholSoxR). The "*" represents absolute conservation, and the "^" a high degree of conservation. The four conserved cysteines provide ligands to the redox active 2Fe–2S cluster.



SCHEME 2. Model for SoxR activation and deactivation.

when the [2Fe–2S] clusters are chemically oxidized under anaerobic conditions [17, 18]. Since oxidation states of metalloproteins in general, and [2Fe–2S] clusters in particular, can be readily analyzed by EPR spectroscopy, this technique has been used extensively in the study of the SoxR protein. *In vivo* EPR measurements of the SoxR [2Fe–2S] clusters demonstrated that the SoxR [2Fe–2S] clusters are predominantly in the reduced state during normal aerobic growth [19–21] and become oxidized when cells are exposed to paraquat or other redox-cycling agents [21]. These results show that the [2Fe–2S] cluster is the redox switch in the SoxR activation process (Scheme 2).

The kinetics of SoxR [2Fe–2S] cluster oxidation and reduction in cells overproducing SoxR has also been determined by EPR spectroscopy [21]. Rapid oxidation (2 min) of SoxR [2Fe–2S] clusters was observed upon cell exposure to any of several structurally unrelated redox- cycling compounds that generate intracellular superoxide under aerobic conditions, including paraquat, menadione, or phenazine methosulfate. Removal of the oxidative stress by preventing aeration led to very rapid re-reduction of the SoxR [2Fe–2S] clusters, such that the protein was mostly reduced within 5 min for paraquat- or phenazine methosulfate-treated cells. The *in vivo* kinetics of SoxR [2Fe–2S] cluster oxidation and reduction paralleled the increase and decrease of transcription of *soxS*, the target gene for SoxR. The *soxS* transcript was induced significantly within 2 min and reached a steady state 100-fold higher than the level in non-stressed cells within 10 min after aerobic exposure to paraquat. Moreover, when the oxidative stress was alleviated by stopping aeration, the amount of *soxS* mRNA in the cells declined rapidly, nearly reaching the low level observed in non-stressed bacteria by 20 min.

What is the direct oxidant that reacts with the [2Fe–2S] cluster in SoxR? The *soxRS* regulon is induced by redox-cycling reagents that are capable of generating superoxide. This process is accompanied by the consumption of cellular reductants, raising the question as to whether the SoxR protein responds directly to superoxide or indirectly to the depletion of NADPH. Experimental results supporting both views have been reported, but no consensus has been reached. In addition, nitric oxide activation of the SoxR protein might occur through yet another different mechanism that remains to be understood.

One important question that has not been resolved is how the reduced state of SoxR is maintained to counter autooxidation during normal aerobic growth. The redox potential of the [2Fe–2S] cluster was determined to be −285 mV, suggesting that the reduction of SoxR may be linked to the NADPH/$NADP^+$ redox pool, which has an estimated redox potential of −340 mV. Since *fpr* (NADPH:flavodoxin oxidoreductase), *fld*A (flavodoxin 1, an iron–sulfur protein reductase), and *zwf* (glucose 6-phosphate dehydrogenase, and NADPH-generating enzyme) are targets of SoxRS regulation, it is attractive to speculate that the oxidized SoxR is reduced by the NADPH:flavodoxin oxidoreductase / flavodoxin 1 couple with the consumption of NADPH, making SoxRS regulation autoregulated. More work is clearly needed to further elucidate the oxidation and reduction mechanisms for the SoxR protein.

OTHER MODES OF REDOX SENSING

As additional redox-sensing proteins are identified and characterized, new modes of redox sensing will certainly emerge. One example is the PerR transcriptional regulator found in the gram-positive bacterium *Bacillus subtilis*. PerR positively regulates the following genes in response to hydrogen peroxide or metal ion limitation: *kat*A (catalase), *ahpCF* (alkyl hydroperoxide reductase), *mrg*A (protective DNA-binding protein homologous to *E. coli* Dps), *hem*AX-*CDBL* (heme biosynthesis operon) [22, 23]. The 21 kDa

PerR protein shares sequence homology with the *E. coli* ferric uptake regulation protein Fur. Like other Fur family members, PerR has a helix-turn-helix DNA-binding motif in its N-terminal region, and contains two CXXC motifs in the C-terminal putative metal-binding domain. Based on sequence homology, it was speculated that PerR is a metalloprotein and that the bound metal ion may play a key role in peroxide sensing. Redox sensing by PerR may be achieved by metal-catalyzed oxidation reactions that could modify the protein or by a change in the oxidation state of the bound metal ion [22]. The mechanism of peroxide sensing by PerR thus may represent a different mode from that of OxyR.

CONCLUSION

In this review, we have described three redox-sensing transcription factors found in prokaryotic cells. The chemical principles learned from the studies of OxyR, SoxR, and PerR should be applicable to eukaryotic systems. For instance, we and others have proposed that the thiol–disulfide switch seen in OxyR may very well be a general mechanism for the regulation of protein activity [6, 24, 25]. The dual roles of sensing divalent metal ions and sensing peroxides exhibited by the PerR protein is reminiscent of similar properties shown by the eukaryotic p53 [26] and MTF1 [27] transcription factors. The methods established through the study of prokaryotic redox-sensing proteins are also likely to be applicable to more complex systems. The ability to characterize the redox-sensing proteins directly using biochemical and biophysical techniques combined with the use of molecular biology and genetic approaches has greatly facilitated the study of prokaryotic regulators. Since the effects of oxidation and reduction can easily be obscured by complex reactions that can take place between reactive oxygen species and cellular constituents, multiple approaches are needed to elucidate the redox regulation occurring inside the cell.

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