OxyR2 Functions as a Three-state Redox Switch to Tightly Regulate Production of Prx2, a Peroxiredoxin of Vibrio vulnificus*

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Ye-Ji Bang‡§, Zee-Won Lee‡§, Dukyun Kim‡§, Inseong Jo§, Nam-Chul Ha‡§, and Sang Ho Choi‡§‡

From the ‡National Research Laboratory of Molecular Microbiology and Toxicology and the §Department of Agricultural Biotechnology, and Center for Food Safety and Toxicology, Seoul National University, Seoul 08826, Korea

The bacterial transcriptional regulator OxyR is known to function as a two-state redox switch. OxyR senses cellular levels of H₂O₂ via a “sensing cysteine” that switches from the reduced to a disulfide state upon H₂O₂ exposure, inducing the expression of antioxidant genes. The reduced and disulfide states of OxyR, respectively, bind to extended and compact regions of DNA, where the reduced state blocks and the oxidized state allows transcription and further induces target gene expression by interacting with RNA polymerase. *Vibrio vulnificus* OxyR2 senses H₂O₂ with high sensitivity and induces the gene encoding the antioxidant Prx2. In this study, we used mass spectrometry to identify a third redox state of OxyR2, in which the sensing cysteine was overoxidized to S-sulfonated cysteine (Cys-SO₃H) by high H₂O₂ in vitro and in vivo, where the modification deterred the transcription of prx2. The DNA binding preferences of OxyR2_C206D, which mimics overoxidized OxyR2, suggested that overoxidized OxyR2 binds to the extended DNA site, masking the −35 region of the prx2 promoter. These combined results demonstrate that OxyR2 functions as a three-state redox switch to tightly regulate the expression of prx2, preventing futile production of Prx2 in cells exposed to high levels of H₂O₂ sufficient to inactivate Prx2. We further provide evidence that another OxyR homolog, OxyR1, displays similar three-state behavior, inviting further exploration of this phenomenon as a potentially general regulatory mechanism.

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1 To whom correspondence may be addressed. Tel.: 82-2-880-4853; Fax: 82-2-873-5095; E-mail: hanc210@snu.ac.kr.

2 To whom correspondence may be addressed. Tel.: 82-2-880-4857; Fax: 82-2-873-5095; E-mail: choish@snu.ac.kr.

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*The abbreviations used are: Prx, peroxiredoxin; AMS, 4-acetamido-4-[(imidazol-1-yl)methyl]-1,2-disulfonic acid.*
Sensing Cysteine of OxyR2 Is Overoxidized at High Levels of H2O2—The purified OxyR2 protein was reacted with 500 μM H2O2 in vitro, and the redox state of the sensing cysteine Cys206 was subjected to MALDI-TOF MS analysis in positive and negative ion reflector modes (Fig. 1). Peptides can be detected in positive and/or negative ion reflector modes, depending on the intrinsic properties of the peptides that influence the ionization behavior (18). Mass spectra for the tryptic digests of the OxyR2 protein were analyzed after the free cysteine residues in the sample were alkylated with iodoacetamide to prevent further oxidation during the analysis. As shown in Fig. 1A, the peptide fragment containing both redox-sensitive cysteine residues (EHC206LTEHAVSAC215K) was detectable in the negative ion reflector mode and had a singly charged monoisotopic mass [M − H]− of 1539.6 regardless of the presence of H2O2 (Fig. 1A, top). However, upon reaction with 500 μM H2O2, the monoisotopic mass [M − H]− of the peptide fragment decreased to 1530.5, suggesting that the peptide fragment contains one S-sulfonated cysteine (Cys-SO3H) and one alkylated cysteine. The peptide fragment with S-sulfonated cysteine (Cys-SO3H) and one alkylated cysteine (Cys-SO3H) was not detectable in the positive ion mode regardless of H2O2 treatment (Fig. 1A, right panels).

To determine which cysteine residue was S-sulfonated by H2O2, the same analysis with the OxyR2-C206S variant was performed in the absence and presence of 500 μM H2O2. The tryptic peptide fragment (EHS206LTEHAVSAC215K) was detected in the positive ion reflector mode and had a singly alkylated protein at Cys215 showing the monoisotopic mass [M + H]+ of 1548.6 regardless of the presence of H2O2 (Fig. 1B, right panels). Neither peptide fragment with Cys215-SO3H ([M + H]+ of 1549.6 or [M − H]− of 1457.6) nor with Cys215-SO2H ([M + H]+ of 1443.6 or [M − H]− of 1441.6) was found,
Three-state Redox Switch OxyR2

![Diagram A](image1)

![Diagram B](image2)

![Diagram C](image3)

FIGURE 2. Overoxidation of OxyR2 Cys206 in V. vulnificus cells exposed to various levels of H₂O₂. A, OH0703 (pDY1025) was grown anaerobically to an A₆₀₀ of 0.3 and exposed to various H₂O₂ concentrations for 3 min. Cellular proteins were precipitated with TCA and alkylated with fresh AMS buffer for 1 h at 37 °C. Proteins (3.5 μg for the top panel and 7 μg for the bottom panel) were resolved by non-reducing SDS-PAGE and immunoblotted using anti-OxyR2 antibody (top) or anti-OxyR2-Cys206-SO₃H antibody (bottom). The predicted numbers of AMS that alkylated each OxyR2 molecule and their redox states are indicated at the ends of the gel. Negative control (NC) was OH0703 (pJH0311), 6AMS control was OH0703 (pBANG1416), and 7AMS control was OH0703 (pDY1025). B, the specificity of anti-OxyR2-Cys²⁰⁶-SO₃H antibody to overoxidized and reduced OxyR2 was determined using ELISA. The microtiter 96-well plates were coated with 0.1 μg of synthetic peptides corresponding to either OxyR2 active site with overoxidized (S-sulfonated) Cys²⁰⁶ (KEHC²⁰⁶(SO₃H)LTEHA) (●) or with reduced Cys²⁰⁶ (KEHC²⁰⁶[SH]LTEHA) (◇), and the peptides were reacted with various concentrations of the antibody as indicated. C, the S-sulfonated OxyR2 peptide (0.1 μg) was attached to the microtiter 96-well plates and then reacted with 4 μg of anti-OxyR2-Cys²⁰⁶-SO₃H antibody. As a binding competitor, either S-sulfonated or reduced OxyR2 peptides (12.5 ng) were added to the reaction with the antibody as indicated. Black bar, control where no competitor was added. Relative binding of the antibody to the specific peptides is presented as A₄₅₀. All data in B and C represent mean ± S.D. (error bars).

indicating that Cys²¹⁵ did not undergo the overoxidation. The EHS²⁰⁶LTEHAVSAC²¹⁵K peptide fragment was not detectable in the negative ion mode (Fig. 1B, left panels). Therefore, the combined results demonstrated that the sensing cysteine, Cys²⁰⁶, but not the other cysteine, Cys²¹⁵, of OxyR2 is overoxidized to Cys-SO₃H in the presence of high levels of H₂O₂ in vitro.

Overoxidation of OxyR2 in the V. vulnificus Cells Exposed to H₂O₂—To ascertain whether the overoxidation of OxyR2 at the sensing cysteine indeed occurs cellular H₂O₂ in V. vulnificus, the redox state of cellular OxyR2 was analyzed. The OxyR2 mutant OH0703 with pDY1025 expressing oxyR2 was grown anaerobically, exposed to various concentrations of H₂O₂, and then treated with 0.5-kDa 4-acetamido-4′-maleimidystilbene-2, 2′-disulfonic acid (AMS; Invitrogen) to alkylate free thiols in the proteins. The total cellular proteins (3.5 μg/well) were resolved on non-reducing SDS-PAGE and immunoblotted with anti-OxyR2 antibody. Alkylation of a free thiol in the proteins with AMS adds 0.5 kDa of molecular mass (Fig. 2A, top). OxyR2 in the cells without exposure to H₂O₂ existed in the reduced state in which all seven cysteine residues present in OxyR2 were alkylated with AMS (7AMS control). An OxyR2 band containing pentuply alkylated cysteine residues coexisted with the heptuply alkylated OxyR2 band when the cells were exposed to 10 μM H₂O₂, indicating that a part of OxyR2 was oxidized to form a disulfide bond, as observed in the previous report (11). More importantly, a portion of OxyR2 in the cells exposed to H₂O₂ exceeding 30 μM appeared to have sextuply alkylated cysteine residues, indicating that one of the seven cysteine residues of OxyR2 had a single modification, such as an oxidation, that could prevent alkylation by AMS. When determined based on the band intensities, relative amounts of the OxyR2 with the single cysteine modification (or sextuply alkylated) were increased gradually by exposure to higher levels of H₂O₂ (Fig. 2A, top).

To examine whether the OxyR2 with a single cysteine modification observed in the cells exposed H₂O₂ exceeding 30 μM is the OxyR2 with overoxidized Cys²⁰⁶, the total cellular proteins (7.0 μg/well) were resolved on non-reducing SDS-PAGE and immunoblotted with the anti-OxyR2-Cys²⁰⁶-SO₃H antibody (Fig. 2A, bottom). The OxyR2 protein with overoxidized Cys²⁰⁶ was specifically detected in cells exposed to H₂O₂ exceeding 30 μM and increased gradually in the cells exposed to higher levels of H₂O₂. The results indicated that the sensing cysteine of OxyR2 becomes overoxidized in vivo when the V. vulnificus cells are exposed to high levels of H₂O₂, as was demonstrated in vitro (Fig. 1).

Concurrently, to determine the specificity of the anti-OxyR2-Cys²⁰⁶-SO₃H antibody by ELISA, the antibody was tested to react with the Cys²⁰⁶-SO₃H or Cys²⁰⁶-SH peptides that were synthesized and attached to the microtiter 96-well plates. The antibody specifically bound to the Cys²⁰⁶-SO₃H
peptide (Fig. 2B). Furthermore, the binding of the antibody to the attached Cys\(^{206}\)-SO\(_3\)H peptide was effectively inhibited by the Cys\(^{206}\)-SO\(_3\)H peptide added to the reaction as a competitor (Fig. 2C). The antibody slightly bound to the Cys\(^{206}\)SH peptide (Fig. 2B), and the Cys\(^{206}\)SH peptide competitor could marginally inhibit the antibody reaction to the Cys\(^{206}\)SO\(_3\)H peptide (Fig. 2C), reflecting that Cys\(^{206}\)SH peptide could be oxidized to Cys\(^{206}\)-SO\(_3\)H peptide in the aerobic conditions we tested. The results indicated that the anti-OxyR2-Cys\(^{206}\)-SO\(_3\)H antibody we used is specific to the OxyR2 with Cys\(^{206}\)-SO\(_3\)H. However, it was not possible to examine the antibody’s specificity to Cys\(^{206}\)SH. Therefore, the OxyR2 with Cys\(^{206}\)-SO\(_3\)H is hereafter designated as the oxidized state of OxyR2.

**Overoxidized State of OxyR2 Turns Off the prx2 Promoter**

To examine the transcriptional activity of the oxidized state of OxyR2, the expression of prx2 was monitored in the wild-type *V. vulnificus* cells. The expression of prx2 was significantly induced when the anaerobically grown cells were exposed to 10 \(\mu\)M \(\text{H}_2\text{O}_2\) (Fig. 3A), indicating that OxyR2 was oxidized to the disulfide state under this condition to activate the prx2 promoter (Prx2) (Fig. 2A). However, the level of prx2 transcript gradually decreased along with increasing concentrations of \(\text{H}_2\text{O}_2\) exceeding 30 \(\mu\)M (Fig. 3A), which was in accordance with the increase of the oxidized state of OxyR2 (Fig. 2A). The changes in prx2 expression were dependent on OxyR2 activity, but not on oxyR2 expression level, because the expression level of oxyR2 was not significantly affected by increasing concentrations of \(\text{H}_2\text{O}_2\) below 1 mM (Fig. 3A). The combined results suggested that the oxidized state of OxyR2 deters further expression of prx2. Because it has been reported that Prx2 is inactivated by \(\text{H}_2\text{O}_2\) exceeding 30 \(\mu\)M (17), this deterrence of the prx2 expression may prevent the worthless production of Prx2 in the environments of high \(\text{H}_2\text{O}_2\), where Prx2 is no longer functional.

However, it was still possible that \(\text{H}_2\text{O}_2\) over 30 \(\mu\)M can also detrimentally affect other transcription factors, such as components of RNA polymerase, to deter the prx2 expression. To rule out this possibility, OH0703 containing pBANG1416 expressing OxyR2-C206D was grown aerobically without exogenously added \(\text{H}_2\text{O}_2\), and the prx2 expression was compared. It has been reported that the size and the polar properties of aspartic acid are comparable with those of the overoxidized cysteine residues (Cys-SO\(_3\)H and Cys-SO\(_2\)H), and therefore OxyR2-C206D is anticipated to mimic the oxidation state of OxyR2 (10). As shown in Fig. 3B, OH0703 producing wild-type OxyR2 expressed almost 13-fold greater prx2 than the negative control strain lacking OxyR2. This supported our previous observation that wild-type OxyR2 activates the prx2 expression under aerobic conditions (11). In contrast, the prx2 expression level of the strain producing OxyR2-C206D was almost the same as that of the negative control strain (Fig. 3B). The results indicated that the deterrence of the prx2 expression in response to \(\text{H}_2\text{O}_2\) exceeding 30 \(\mu\)M is caused mainly, if not solely, by overoxidation of OxyR2 and further confirmed that OxyR2-C206D is functionally similar to the oxidized state of OxyR2.

**OxyR2 Regulates prx2 as a Three-state Redox Switch in Response to \(\text{H}_2\text{O}_2\)**

Fig. 3C represents the working model of OxyR2 that regulates the expression of prx2 as a three-state redox switch. OxyR2 adopts the three distinct redox states (reduced state, disulfide state, and oxidized state) in an \(\text{H}_2\text{O}_2\) concentration-dependent manner (Figs. 1 and 2). So far in the widely accepted mechanism of OxyR proteins, OxyR2...
Three-state Redox Switch OxyR2

![Diagram of OxyR2 binding sites](image)

FIGURE 4. Binding sequences of OxyR2SCA-C206D within the prx2 promoter region. A, a 260-bp DNA fragment of the upstream region of Pprx2 was radioactively labeled and then used as probe DNA. The radiolabeled probe DNA (25 nM) was incubated with increasing amounts of the purified OxyR2SCA-C206D as indicated and then digested with DNase I. The regions protected by OxyR2SCA-C206D are indicated by open boxes, whereas the nucleotides showing enhanced cleavage are indicated by a black box. Lanes C, T, A, and G, nucleotide sequencing ladder of Pprx2. Nucleotide numbers shown are relative to the transcription start site of Pprx2. B, the sequences for binding of OxyR2SCA-C206D are indicated below the Pprx2 sequences as diagonal-filled boxes. The sequences for binding of oxidized OxyR2 (white box) and reduced OxyR2 (gray boxes) determined previously (11) are presented above the Pprx2 sequences. The nucleotides showing enhanced cleavage by binding of OxyR2SCA-C206D and the reduced OxyR2 are indicated as black boxes. The transcription start site of Pprx2, identified previously (11) is indicated by a bent arrow, and the positions of the putative −10 and −35 regions are underlined. The ATG translation initiation codon and the putative ribosome-binding site (SD) are also indicated in boldface type.

shifts its redox state from the reduced state to the disulfide state upon reaction with H2O2 and activates the expression of prx2. However, when exposed to H2O2 exceeding the working range for Prx2, OxyR2 moves to an oxidized state, a third redox state, and turns off the expression of prx2. It is obvious that OxyR2 is able to prevent production of useless Prx2 and thus save valuable cellular resources by working as a three-state redox switch (Fig. 3C).

Mechanism of the Oxidized OxyR2 to Turn Off Pprx2—To gain insight into the mechanism by which the oxidized state of OxyR2 deters the expression of prx2, the sequences for binding of the oxidized OxyR2 were determined. Purified OxyR2SCA-C206D, which mimics oxidized OxyR2 and is soluble under aerobic conditions, was used in vitro for the DNA footprinting assay. OxyR2SCA, in which all five non-catalytic cysteine residues in OxyR2 were replaced with alanine residues, was constructed previously (11). The purified OxyR2SCA did not form oligomers in vitro under nonreducing conditions (11). As shown in Fig. 4, the sequences for binding of OxyR2SCA-C206D were extended from −80 to −26 relative to the transcription start site of Pprx2. Therefore, one possible mechanism is that the oxidized OxyR2 presumably turns off the expression of prx2 by masking the −35 region and thereby preventing RNA polymerase binding to Pprx2.

Furthermore, the binding sequences of OxyR2SCA-C206D are identical to those of the reduced OxyR2. Enhanced cleavage in several nucleotides (−53 to −55), which was observed in the binding sequences of the reduced OxyR2, was also found in the binding sequences of OxyR2SCA-C206D (Fig. 4B). It has been proposed that OxyR tetramer in the reduced state adopts an extended conformation (10) and binds to the elongated DNA-binding sites (11, 12). The combined results suggest that the oxidized state of OxyR2 turns off the activation of prx2 by adopting a conformation similar to that of the reduced state, binding to the elongated sequences, and preventing RNA polymerase binding to the promoter sequences.

Possible Overoxidation of OxyR1—To probe whether other OxyR proteins could function as a three-state redox switch, the expression of prx1 and katG in the V. vulnificus cells exposed to various levels of H2O2 was determined (Fig. 5, A and B). It has been reported that OxyR1, a homolog of E. coli OxyR, directly activates the expression of prx1 and katG (11). Expression of both antioxidant genes in V. vulnificus increased along with increasing levels of H2O2, but decreased when H2O2 levels exceeded certain levels; the expression levels of prx1 and katG showed the highest peaks in cells exposed to 30 and 10 μM H2O2, respectively, and then decreased in cells exposed to higher levels of H2O2 (Fig. 5, A and B). This variation of the prx1 and katG expression in response to increasing levels of H2O2 was similar to that of the prx2 expression (Fig. 3A), indicating that OxyR1 was also probably overoxidized to turn off expression of prx1 and katG when the H2O2 levels exceeded the working ranges for Prx1 and KatG. This result suggested that OxyR homologs of other bacteria could function as a three-state redox switch to tightly regulate their target antioxidant genes.

Discussion

In this study, we present evidence that V. vulnificus OxyR2 regulates the expression of prx2 as a three-state redox switch. When OxyR2 encountered H2O2 exceeding the Prx2 working range, the sensing cysteine of OxyR2 was oxidized to Cys206-SO2H in vivo as well as in vitro (Figs. 1 and 2). Although MS analysis was unable to detect the peptide fragment of OxyR2 with Cys206-SO2H (Fig. 1A), the S-sulfonated form of OxyR2 was most likely formed as an intermediate in the course of the overoxidation of OxyR2 to the S-sulfonated OxyR2 (19).
In addition to the reduced and oxidized (disulfide) states, the overoxidized state is the third redox state of OxyR2, whose function was primarily characterized in this study. The overoxidized OxyR2 actively turns off the transcription of prx2 by binding to elongated sequences masking the -35 region of Prx2 in the environments of high H2O2, where Prx2 is no longer functional (Figs. 3 and 4) (17). Obviously, the overoxidized OxyR2 can prevent production of useless Prx2 and thus save valuable cellular resources. Therefore, the overoxidized OxyR2 is not simply an expired or perished form of OxyR2 but rather plays an important role in the survival of V. vulnificus during pathogenesis.

It was noteworthy that expression of prx1 and katG was increased, reached a maximum, and then was decreased by exposure of cells to increasing levels of H2O2 in a pattern similar to that of prx2 (Fig. 5, A and B). Both prx1 and katG are regulated by OxyR1, a homolog of E. coli OxyR (11), implying that OxyR2 is not the only OxyR regulating antioxidant genes as a three-state redox switch. OxyR has been primarily thought of as a two-state redox switch that turns the antioxidant genes off and on in an H2O2-dependent manner (7–9). When bacteria encounter manageable ranges of H2O2, OxyR turns on its target genes encoding antioxidants, such as Prxs and catalases. Scavenging the H2O2 using the antioxidants and maintaining metabolic activities to keep growing is beneficial for bacteria. Accordingly, expression of most of the OxyR regulon is governed by the housekeeping σ factor RpoD (σ^{32}) (20). However, when H2O2 exceeds the manageable ranges, maintaining expressions of the antioxidant genes would be worthless because growth and proliferation of bacterial cells under this condition could be reckless. Instead, bacteria rather express many stress tolerance genes governed by the stress-responsive σ factor RpoS (σ^{38}) and shift their global physiology to stationary (or dormant) phase (21, 22). Bacteria can turn off the antioxidant genes using OxyR, as a three-state redox switch, under conditions in which expression of the antioxidants is useless. It is obviously advantageous that bacteria utilize more cellular resources to express stress tolerance genes encoding many additional protective systems (20, 21).

The mechanism for the overoxidation of the sensing cysteine remains unclear. However, structural analysis of the Pseudomonas aeruginosa OxyR-C199D mutant, in which the sensing cysteine residue is replaced with aspartic acid, gives us a hint as to this mechanism (10). The crystal structure OxyR-C199D holds a H2O2 molecule near aspartic acid that is even bulkier than a cysteine residue, implying that H2O2 can also bind to the same site even after the sensing cysteine is oxidized to Cys-SOH or Cys-SO_2_3H. The bound H2O2 can eventually oxidize the cysteine to Cys-SO_3_3H by the same H2O2-driven oxidation mechanism proposed previously (10). We modeled Cys-SOH, Cys-SO_2_3H, and Cys-SO_3_3H forms based on the P. aeruginosa OxyR-C199D structure that contains H2O2 molecules (Fig. 6A). Steric clash was not observed around the modeled cysteine residues with modification. Moreover, the lone pair electrons of the sulfur atoms in the cysteine residues could be facing H2O2 within a reasonable distance for nucleophilic attack on the H2O2 molecule when the -SOH or -SO_2_3H moiety is simply rotated in the model. These findings support the idea that overoxidation at the sensing cysteine residue by H2O2 can occur faster than at other cysteine residues, presumably by the successive H2O2-driven oxidation mechanism.

Three different redox states of OxyR are determined, depending on the cellular levels of H2O2 as depicted in Fig. 6B. At low levels of H2O2 below the sensing level of OxyR, OxyR is maintained in the reduced state. Under the Prx2 working range of H2O2, a kinetic path renders the sensing cysteine in OxyR oxidized to the Cys-SOH intermediate that forms a disulfide bond with the other redox-sensitive cysteine, leading to activation of the target antioxidant genes. However, when H2O2 levels exceed the Prx2 working range, the Cys-SOH intermediate would be rapidly overoxidized to the Cys-SO_2_3H via Cys-SO_3_3H by H2O2 before making the disulfide bond. The resulting overoxidized OxyR2 turns off the production of useless Prx2. We do not yet know what happens to the overoxidized OxyR when the cellular H2O2 level returns to the working range. One plausible mechanism is that the overoxidized OxyR is degraded and new OxyR is synthesized, so that the cell can constantly respond to the changing environment. Given that the cellular amount of OxyR2 is very low (11), the energy needed for this process is not high.

In summary, V. vulnificus OxyR2 is primarily characterized as a three-state redox switch. A thiol-based sensor protein OxyR2 senses H2O2 and shifts to a third redox state, the overoxidized state, as a result of overoxidation of sensing cysteine residue by H2O2. Overoxidized OxyR2 prevents production of Prx2 where Prx2 is no longer functional and thereby saves val-
Three-state Redox Switch OxyR

FIGURE 6. Proposed overoxidation mechanism of OxyR. A, modeled structures of OxyR, focusing on the sensing cysteine (Cₕ). S-Hydroxylated sensing cysteine (CS-SOH) is oxidized by the bound H₂O₂ (left), resulting in S-sulfonated sensing cysteine (CS-SO₂H, middle), which is in turn further oxidized to S-sulfated sensing cysteine (CS-SO₃H, right). B, schematic reaction mechanism of oxidation of sensing cysteine and the concomitant three redox states of OxyR. Sensing cysteine of OxyR in the reduced state is converted to S-hydroxylated sensing cysteine by H₂O₂, as a reaction intermediate (black arrow). S-Hydroxylated sensing cysteine rapidly makes a disulfide with the other redox-sensitive cysteine (Cₐ), resulting in the disulfide state of OxyR (blue arrow). Alternatively, S-Hydroxylated sensing cysteine is further oxidized consecutively by H₂O₂ to the final S-sulfonated sensing cysteine in the presence of excess H₂O₂, resulting in the oxidized state of OxyR (red arrows).

uatable cellular resources. The DNA sequences for binding of OxyR²⁻C₂⁰⁶D suggested that overoxidized OxyR² adopts an extended conformation and turns off P₉₀% N₂⁵ % C O₂, and 5% H₂ (Coy Laboratory Products, Grass obtaining using an anaerobic chamber with an atmosphere of 2.0% (w/v) NaCl (LBS) at 30 °C. Anaerobic conditions were strains were grown in LB medium supplemented with significus and plasmids used in this study are listed in Table 1. The Experimental Procedures

Bacterial Strains, Plasmids, and Culture Media—The strains and plasmids used in this study are listed in Table 1. The V. vulnificus strains were grown in LB medium supplemented with 2.0% (w/v) NaCl (LBS) at 30 °C. Anaerobic conditions were obtained using an anaerobic chamber with an atmosphere of 90% N₂, 5% CO₂, and 5% H₂ (Coy Laboratory Products, Grass Lake, MI). For anaerobic culture, the media were preincubated to remove dissolved O₂ in the anaerobic chamber, which was verified by adding 0.00001% (w/v) resazurin salt (Sigma) to the media as described previously (11).

Site-directed Mutagenesis of oxyR² and Purification of Mutant OxyR² Proteins—A mutant OxyR² in which Cys²⁰⁶ was replaced with aspartic acid was constructed using the QuikChange® site-directed mutagenesis kit (Agilent Technologies, Loveland, CO) as described previously (11). The complementary mutagenic primers listed in Table 2 were used in conjunction with the plasmid pDY1025 (oxyR² cloned into a broad host-range vector pJH0311 under the lac promoter) to create pBANG1416 (oxyR²-C₂⁰⁶D on pJH0311) (Table 1). The mutation was confirmed by DNA sequencing. E. coli SM10 λ pir, tra (23) harboring pJH0311, pDY1025, or pBANG1416 was used as a conjugal donor to the oxyR² mutant (OH0703). The conjugation was conducted as described previously (11).

Mutant OxyR² proteins, OxyR²-C₂⁰⁶S and OxyR²₅Cₐ⁻C₂⁰⁶D, were constructed and purified for MALDI-TOF MS analysis and DNA footprinting assay, respectively. Cys²⁰⁶ of OxyR² was replaced with serine using pDY1001 (oxyR² on pET-28a(+) as a template) and the complementary mutagenic primers (Table 2) to result in pDY1104 (oxyR²-C₂⁰⁶S on pET-28a(+)). Similarly, Cys²⁰⁶ of OxyR²₅Cₐ was replaced with serine using pDY1014 (oxyR²₅Cₐ on pET-28a(+) as a template) to result in pBANG1501 (oxyR²₅Cₐ-C₂⁰⁶D on pET-28a(+) (Table 1). The resulting His₉ₐ-tagged mutant OxyR² proteins were produced in E. coli BL21 (DE3) and purified by affinity chromatography using Ni²⁺-nitrilotriacetic acid resin (Qiagen, Valencia, CA) as described previously (11).

MALDI-TOF MS Analysis—To investigate the redox state of Cys²⁰⁶ in OxyR² in vitro, the full-length His₉ₐ-tagged OxyR² and OxyR²-C₂⁰⁶S proteins were analyzed by MALDI-TOF MS, as described previously (11, 17). Briefly, to reduce the proteins, 20 μg of proteins in a reaction buffer (20 mM Tris (pH 7.4), 0.3 M KCl, 5 mM MgCl₂, 0.5 mM EDTA, and 10% (v/v) glycerol) were
treated with 100 mM DTT for 1 h, and then the DTT was removed by gel filtration chromatography under anaerobic conditions. To prepare oxidized proteins, reduced OxyR2 proteins were reacted with 500 μM H₂O₂ for 10 min after the DTT removal. Subsequently, the reduced cysteine residues of each reduced and overoxidized OxyR2 protein were alkylated with 50 mM iodoacetamide for 1.5 h in the dark under anaerobic conditions. Alkylated OxyR2 proteins were resolved on non-reducing SDS-PAGE, and the protein bands were excised and in-gel digested with trypsin (Sigma). Peptides were extracted from the gel pieces with 0.1% trifluoroacetic acid in 50% acetonitrile, concentrated to volumes of 10 μl using a SpeedVac concentrator (Savant Instruments Inc., Farmingdale, NY), and desalted using Zip-Tip C₁₈ reverse phase peptide separation matrix (Millipore, Billerica, MA).

MALDI-TOF MS analyses were carried out on a Voyager-DE™ STR biospectrometry work station (Applied Biosystems Inc., Foster City, CA) operating in positive and negative ion reflector modes. α-Cyano-4-hydroxycinnamic acid (10 mg/ml) in 50% acetonitrile and 0.1% trifluoroacetic acid was used as a matrix. The theoretical monoisotopic masses ([M – H]⁻ for the deprotonated form in the negative ion reflector mode or [M + H]⁺ for the protonated form in the positive ion reflector mode) of the cleavage peptides were determined using Peptide-Mass software from the ExPASy proteomics server. The masses from the positive ion reflector mode were calibrated internally with known masses of autolytic trypsin peptides. However, the masses from the negative ion reflector mode were calibrated because the trypsin peaks were not detectable in the negative ion mode.

In Vivo Alkylation of OxyR2 and Western Blotting Analysis—
The oxyR2 mutant OH0703 with pDY1025 expressing oxyR2 was used for Western blotting analysis of OxyR2 as described previously (11). OH0703 (pDY1025) was grown anaerobically to an A₆₀₀ of 0.3, aliquoted to the same volume, and exposed to various concentrations of H₂O₂. To alkylate free thiols in the proteins with AMS, the cells were immediately precipitated with ice-cold TCA, and then the resulting pellets were dissolved in 50 μl of the fresh AMS buffer (15 mM AMS, 1 mM Tris, 1 mM EDTA, 0.1% (w/v) SDS, pH 8.0) (11). After incubation at 37 °C for 1 h, the same amounts of pelleted total protein were resolved on SDS-PAGE under non-reducing conditions and immunoblotted with either anti-OxyR2 or anti-OxyR2-Cys²⁰⁶-SO₃H antibody as described previously (11). The anti-OxyR2 polyclonal antibody was prepared previously (11). The anti-OxyR2 polyclonal antibody was prepared in rabbit by using the purified His₆-tagged OxyR2 as an antigen (11). An S-sulfonated peptide corresponding to the active site of OxyR2, EKEHC²⁰⁶-SO₃H-LTEHA, was synthesized and conjugated with keyhole limpet hemocyanin and then used to raise rabbit anti-OxyR2-Cys²⁰⁶-SO₃H polyclonal antibody (AbFrontier, Seoul, South Korea).

### TABLE 1

**Bacterial strains and plasmids used in this study**

| Strains or plasmids | Relevant characteristics* | Reference or source |
|---------------------|---------------------------|---------------------|
| **Bacterial strains** |                           |                     |
| *V. vulnificus*      |                           |                     |
| MO6-24/O            | Clinical isolate; virulent | Laboratory collection |
| OH0703              | MO6-24/O with oxyR2::nptII; Km² | Ref. 11 |
| **E. coli**          |                           |                     |
| SM10 λ pir          | thi thr leu tna A lac y supE recA::RP4–2 Tc::Mu Apir | Ref. 23 |
| **Plasmids**         |                           |                     |
| pH3011              | 0.3-kb MCS of pUC19 cloned into pCOSS; Ap⁶, Cm⁴ | Ref. 26 |
| pDY1025             | pH3011 with wild-type oyxR2; Ap⁶, Cm⁴ | Ref. 11 |
| pBANG1416           | pH3011 with the mutant oyxR2 encoding OxyR2-²⁰⁶D; Ap⁶, Cm⁴ | This study |
| pET-28a(+)          | His₆-tag fusion protein expression vector; Km² | Novagen |
| pDY1001             | pET-28a (+) with wild-type oyxR2; Km² | Ref. 11 |
| pDY1104             | pET-28a (+) with the mutant oyxR2 encoding OxyR2-²⁰⁶S; Km² | This study |
| pDY1104             | pET-28a (+) with the mutant oyxR2 encoding OxyR2-²⁰⁶D; Km² | This study |
| pBANG1501           | pET-28a (+) with the mutant oyxR2 encoding OxyR2-²⁰⁶D; Km² | This study |

* Ap⁶, ampicillin-resistant; Cm⁴, chloramphenicol-resistant; Km², kanamycin-resistant.

### TABLE 2

**Oligonucleotides used in this study**

| Name                  | Oligonucleotide Sequence (5’→3’)¹,²  | Use                                      |
|-----------------------|--------------------------------------|------------------------------------------|
| **For mutagenesis**   |                                      |                                          |
| OXYR2C206D-F          | GGGAAAAAGAGCATGATCTGACTGAAACACCGGGTGGCG | Construction of OxyR2-²⁰⁶D mutant         |
| OXYR2C206D-R          | CCAGACACCGGCTGTTGCAGTCAGATCATGCTCTTTTCCC |                                          |
| OXYR2C206S-F          | GGGAAAAAGAGCATGATCTGACTGAAACACCGGGTGGCC |                                          |
| OXYR2C206S-R          | GGGCCGCCACACCGGCTGTTGCAGTCAGATCATGCTCTTTTCCC |                                          |
| **For quantitative real-time PCR** |                                      |                                          |
| PRX2QRT-F             | GGGAAAAAGAGCATGATCTGACTGAAACACCGGGTGGCG | Expression of prx2                       |
| PRX2QRT-R             | TCAGATCTGACTGAAACACCGGCTGTTGCAGTCAGATCATGCTCTTTTCCC | Expression of oyxR2                     |
| OXYR2QRT2-F           | GGGAAAAGAGCATGATCTGACTGAAACACCGGGTGGCC | Expression of prx1                       |
| OXYR2QRT2-R           | GGGCCGCCACACCGGCTGTTGCAGTCAGATCATGCTCTTTTCCC | Expression of katG                      |
| PRX1QRT-F             | TGACATCTGACTGAAACACCGGCTGTTGCAGTCAGATCATGCTCTTTTCCC |                                          |
| KATGQRT-F             | CGGGCCACACCGGCTGTTGCAGTCAGATCATGCTCTTTTCCC |                                          |
| KATGQRT-R             | CGGGCCACACCGGCTGTTGCAGTCAGATCATGCTCTTTTCCC |                                          |
| **For DNA footprinting** |                                      |                                          |
| PRX2P02-F             | GGGAAAAAGAGCATGATCTGACTGAAACACCGGGTGGCG | Amplification of prx2 upstream region   |
| PRX2P02-R             | GGGCCGCCACACCGGCTGTTGCAGTCAGATCATGCTCTTTTCCC |                                          |

¹ The oligonucleotides were designed using the *V. vulnificus* MO6-24/O genomic sequence (GenBank™ accession numbers CP002469 and CP002470).
² Regions of oligonucleotides not complementary to the corresponding genes are underlined.
Three-state Redox Switch OxyR2

ELISA—The Cys\textsuperscript{206}-SH peptide EKEHC\textsuperscript{206}(SH)LTEHA and its S-sulfonated form, Cys\textsuperscript{206}-SO\textsubscript{3}H peptide EKEHC\textsuperscript{206}(SO\textsubscript{3}H)-LTEHA, were synthesized (AbFrontier) and used to analyze the binding specificity of anti-OxyR2-Cys\textsuperscript{206}-SO\textsubscript{3}H antibody. The microtiter 96-well plates were coated with 100 μl of each of the synthetic peptides (1 μg/ml) for overnight at 4 °C and blocked with the blocking buffer (2% skim milk in PBS with 20 mM 2-mercaptoethanol) for 2 h to prevent a nonspecific binding. After the blocking buffer was disposed, various concentrations of anti-OxyR2-Cys\textsuperscript{206}-SO\textsubscript{3}H antibody in 100 μl of the blocking buffer were added to each well and reacted for 2 h. When needed, the peptides were added to the reaction along with the antibody as binding competitors.

Then the wells were reacted with HRP-conjugated goat anti-rabbit IgG (0.2 μg/ml) (AbFrontier) for 2 h. For the colorimetric development of HRP, 3,3’5,5’-tetramethylbenzidine solution (0.0075% (w/v), Sigma) was added and reacted for 1 min. The reaction was stopped by adding 100 μl of 2 M H\textsubscript{2}SO\textsubscript{4}. Absorbance at 450 nm (A\textsubscript{450}) was recorded to measure the amount of anti-OxyR2-Cys\textsuperscript{206}-SO\textsubscript{3}H antibody in each well using an Infinite\textsuperscript{TM} M200 microplate reader (Tecan, Männedorf, Switzerland). The wells were washed between each step of the reaction either with TBST-M (50 mM Tris, 150 mM NaCl, 0.1% Tween 20, 20 mM 2-mercaptoethanol) before anti-OxyR2-Cys\textsuperscript{206}-SO\textsubscript{3}H antibody was added or with TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween 20) after the antibody was added. All reactions were carried out at room temperature.

RNA Purification and Transcript Analysis—Total cellular RNAs from the cultures grown anaerobically to an A\textsubscript{600} of 0.3 or aerobically to an A\textsubscript{600} of 0.5 were isolated using RNAprotect\textsuperscript{®} bacteria reagent and an RNeasy\textsuperscript{®} minikit (Qiagen). When necessary, the cultures were exposed to H\textsubscript{2}O\textsubscript{2} for 3 min and then harvested. For quantitative real-time PCR, cDNA was synthesized using the iScript\textsuperscript{TM} cDNA synthesis kit (Bio-Rad), and real-time PCR amplification of the cDNA was performed using the Chromo 4 real-time PCR detection system (Bio-Rad) with pairs of primers listed in Table 2. Relative expression levels of the specific transcripts were calculated using the 16S rRNA expression level as the internal reference for normalization (24).

DNA Footprinting Assay—DNA footprinting assays were conducted as described previously (11). The 260-bp upstream region of P\textsubscript{prx2} extending from −246 to +14, was amplified by PCR using [γ-\textsuperscript{32}P]ATP-labeled PRX2P02-R and unlabeled PRX2P02-F primers (Table 2). The labeled 260-bp DNA (25 nm) probe was incubated with various concentrations of purified OxyR2\textsubscript{SCA-C}206D for 30 min at 30 °C in a 20-μl reaction mixture containing 1× binding buffer (25 mM Tris-Cl (pH 8.0), 25 mM KCl, 6 mM MgCl\textsubscript{2}, 0.5 mM EDTA, 10% (v/v) glycerol, 0.05% (v/v) Tween 20, and 50 μg/ml BSA). The DNA-protein complexes were digested with 0.1 units of DNase I for 30 s at 30 °C. After ethanol precipitation, the digested DNA products were resolved on a sequencing gel alongside sequencing ladders of the same 260-bp upstream region of P\textsubscript{prx2}. The gels were visualized using a phosphor imager analyzer (BAS1500, Fuji Photo Film Co. Ltd., Tokyo, Japan).

Modeling of OxyR2 Structures—To construct models for an overoxidized OxyR2 structure containing a H\textsubscript{2}O\textsubscript{2} molecule, we used the coordinates of the full-length OxyR-C199D variant from P. aeruginosa (Protein Data Bank code 4X6G), where an H\textsubscript{2}O\textsubscript{2} molecule is bound near the mutated Asp\textsuperscript{199} residue corresponding to Cys\textsuperscript{206} of OxyR2. The Asp\textsuperscript{199} residue in the structure was changed to Cys-SOH, Cys-SO\textsubscript{3}H or Cys-SO\textsubscript{3}H using the program COOT (25). Then -SOH, -SO\textsubscript{2}H, or -SO\textsubscript{3}H was attached to the program LITEHA, were synthesized (AbFrontier) and used to analyze the binding of anti-OxyR2-Cys\textsuperscript{206}-SO\textsubscript{3}H. The wells were washed between each step of the reaction to prevent a nonspecific binding.

Data Analyses—Mean and S.D. were calculated from at least three independent experiments. To analyze H\textsubscript{2}O\textsubscript{2} concentration-dependent expression of prx2, oxyr2, prx1, and katG, data were assessed for normal distribution and homogeneity of variance by Shapiro- Wilks’ test. Data that had normal distribution were analyzed by one-way analysis of variance, and data that did not have normal distribution were analyzed by Kruskal-Wallis one-way analysis of variance on ranks. Statistical significance was assessed by the Student-Newman-Keuls method as a post hoc test using SigmaPlot version 13. Significance of differences between experimental groups was accepted at a p value of <0.05.

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