The hydrogen peroxide hypersensitivity of OxyR2 in *Vibrio vulnificus* depends on conformational constraints

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Most Gram-negative bacteria respond to excessive levels of H$_2$O$_2$ using the peroxide-sensing transcriptional regulator OxyR, which can induce the expression of antioxidant genes to restore normality. *Vibrio vulnificus* has two distinct OxyRs (OxyR1 and OxyR2), which are sensitive to different levels of H$_2$O$_2$ and induce expression of two different peroxidases, Prx1 and Prx2. Although OxyR1 has both high sequence similarity and H$_2$O$_2$ sensitivity comparable with that of other OxyR proteins, OxyR2 exhibits limited sequence similarity and is more sensitive to H$_2$O$_2$. To investigate the basis for this difference, we determined crystal structures and carried out biochemical analyses of OxyR2. The determined structure of OxyR2 revealed a flipped conformation of the peptide bond before Glu-204, a position occupied by glycine in other OxyR proteins. Activity assays showed that the sensitivity to H$_2$O$_2$ was reduced to the level of other OxyR proteins by the E204G mutation. We solved the structure of the OxyR2-E204G mutant with the same packing environment. The structure of the mutant revealed a dual conformation of the peptide bond before Gly-204, indicating the structural flexibility of the region. This structural duality extended to the backbone atoms of Gly-204 and the imidazole ring of His-205, which interact with H$_2$O$_2$ and invariant water molecules near the peroxidatic cysteine, respectively. Structural comparison suggests that Glu-204 in OxyR2 provides rigidity to the region that is important in H$_2$O$_2$ sensing, compared with the E204G structure or other OxyR proteins. Our findings provide a structural basis for the higher sensitivity of OxyR2 to H$_2$O$_2$ and also suggest a molecular mechanism for bacterial regulation of expression of antioxidant genes at divergent concentrations of cellular H$_2$O$_2$.

Most aerobic organisms encounter reactive oxygen species, including hydrogen peroxide (H$_2$O$_2$), during aerobic respiration processes (1, 2). At higher than critical concentrations of H$_2$O$_2$, highly toxic hydroxyl radicals, which can damage essential cellular components, can be generated (3). To cope with H$_2$O$_2$ stress, many bacteria have developed elaborate sensing and scavenging systems. In particular, pathogenic bacteria have sophisticated systems to overcome host immune responses that produce a high level of H$_2$O$_2$ (4).

Many LysR-type transcriptional regulators are found in various bacteria, and they are typically homotetramers that contain an N-terminal DNA-binding domain (DBD) and C-terminal regulatory domain (RD) (5). OxyR, a member of the LysR-type transcriptional regulator family, recognizes H$_2$O$_2$ above a certain threshold concentration and induces a diverse array of antioxidant genes, including catalase-peroxidase katG and peroxiredoxins (prx genes) (6, 7). OxyR has a DBD for recognizing target gene promoters and an RD containing two conserved cysteine residues (peroxidatic and resolving cysteines) for sensing H$_2$O$_2$ (8). Many crystal structures have been reported since the first descriptions of *Escherichia coli* OxyR structures revealed the redox-state-dependent conformational change of RDs (8). Recently elucidated full-length OxyR crystal structures from *Pseudomonas aeruginosa* have revealed the homotetrameric arrangement of the protein, where the two dimeric RDs face each other, and the two dimeric DBDs are oriented in the same direction. The crystal structures have also provided insight into how this redox-dependent structural alteration of RDs is propagated to the DBDs, leading to inward or outward motion between dimeric DBDs (9).

Reduced OxyRs that have two free thiols in the two conserved cysteine residues are converted into oxidized forms via two steps in the presence of H$_2$O$_2$ (10, 11). The peroxidatic cysteine residue of OxyR is first oxidized by H$_2$O$_2$ to cysteine-sulfenic acid (Cys-SOH), in which a bound H$_2$O$_2$ molecule and two water molecules near the peroxidatic cysteine are thought to play crucial roles via a so-called “H$_2$O$_2$-driven oxidation mechanism” (9). The Cys-SOH ultimately forms a disulfide bond via the resolving cysteine in a certain concentration range.
of \( H_2O_2 \), resulting in formation of the oxidized (or disulfidized) form of OxyR that activates the induction of target genes (11, 12).

*Vibrio vulnificus* is a facultative aerobic pathogen that can cause fatal septicemia (13, 14) and has two different OxyR proteins (VvOxyR1 and VvOxyR2) and two different Prx proteins (VvPrx1 and VvPrx2) (15, 16). *V. vulnificus* has high sequence and functional similarities to typical types of OxyR proteins and induces VvPrx1 expression at a certain \( H_2O_2 \) concentration (15.9%) (16). VvOxyR2 exhibits limited sequence similarity to other typical OxyR types and is activated by a lower \( H_2O_2 \) concentration (~0.5 \( \mu M \)) than VvOxyR1. VvOxyR2 induces VvPrx2 encoding VvPrx2, which has higher activity than VvPrx1 at low \( H_2O_2 \) levels, although VvPrx2 is more susceptible to irreversible inactivation caused by overoxidization of the peroxidatic cysteine above the working concentration of \( H_2O_2 \) (17). Therefore, it was concluded that VvOxyR2 is also a dimer in solution data set. The asymmetric unit contained two protomers of VvOxyR2-RD, and the final model produced 19.2\% R-factor with reasonable stereochemistry (Table 1). Like other OxyR-RDs, the protomer consists of two subdomains (RD-I and RD-II; Fig. 1A). Two notable dimers generated by the crystallographic 2-fold axis were found in the crystal, where each protomer individually participated in dimer formation. These observations indicate that VvOxyR2 is also a dimer in solution, as are other OxyR proteins. The overall structure of VvOxyR2-RD was similar to other OxyR-RD structures previously determined in the reduced state (Fig. 1, A and B). The two conserved cysteine residues (Cys-206 and Cys-215) remained in the reduced state (8, 9, 18, 19). Thus, it was concluded that VvOxyR2-RD exhibited the typical reduced conformation of OxyR.

To analyze the structure in more detail, we superposed the VvOxyR2-RD structure on *P. aeruginosa* OxyR (PaOxyR) in the reduced state (Fig. 1B). Whereas the RD-I subdomain showed minimal deviation among the structures, the RD-II subdomain had a particular variation in the location of the resolving cysteine (Cys-215 in VvOxyR2, Cys-208 in PaOxyR) (Fig. 1C). Compared with cysteine positions in other OxyR proteins, the resolving cysteine Cys-215 is buried in the interior of the RD-II

### Table 1

| VvOxyR2-RD WT (SO4-free) | WT (ZnCl2-soaked) | E204G (SO4-free) (high resolution) | E204G (SO4-free) (P3, 21) | E204G (SO4-bound) |
|--------------------------|-------------------|-----------------------------------|--------------------------|------------------|
| Data collection          |                   |                                   |                          |                  |
| Beam line                | PAL 5C            | PAL 5C                            | PAL 5C                   | PAL 7A           |
| Wavelength (Å)           | 0.97940           | 1.28230                           | 1.00820                  | 0.97933          |
| Space group              | C222, C222        | C222, C222                         | C222, P3, 21             | C222, C222       |
| Cell dimensions          |                   |                                   |                          |                  |
| a, b, c (Å)              | 70.2, 138.3, 97.5 | 70.3, 138.4, 97.4                 | 71.2, 138.2, 97.1        | 71.2, 138.2, 97.1 |
| α, β, γ (degrees)        | 90, 90, 90        | 90, 90, 90                         | 90, 90, 120              | 90, 90, 90       |
| Resolution (Å)           | 50.0–1.60 (1.63–1.60)* | 50.0–1.96 (1.99–1.96)              | 50.0–1.55 (1.58–1.55)    | 50.0–1.55 (1.58–1.55) |
| R因子 (Å)                | 0.027 (0.164)     | 0.034 (0.113)                      | 0.028 (0.163)            | 0.089 (0.368)    |
| f/σ (σ)                  | 18.6 (2.45)       | 51.2 (12.6)                        | 19.3 (3.2)               | 9.7 (2.46)       |
| Completeness (%)         | 98.7 (95.5)       | 99.6 (99.4)                        | 99.4 (96.0)              | 96.6 (88.0)      |
| Redundancy               | 8.0 (4.2)         | 5.4 (4.7)                          | 9.5 (6.2)                | 4.8 (3.1)        |

*Values in parentheses are for the highest-resolution shell.*
subdomain along with the inter-cysteine α-helix. However, the peroxidatic cysteine is located at the same position as in other OxyR proteins (Fig. 1).

VvOxyR2 has a noncanonical conformation of the peptide bond between Lys-203 and Glu-204

Notably, the structural superposition of VvOxyR2 with other OxyR proteins revealed large conformational differences at the peptide bond (or peptide flipping) between Lys-203 and Glu-204 (Fig. 1C). However, neither the Lys-203 nor Glu-204 side chains was involved in an interaction with other residues or the bound H₂O₂ and water molecules near the peroxidatic cysteine residue. Sequence alignment revealed that Glu-204 of VvOxyR2 was replaced with a glycine residue observed in most OxyR proteins and that the glycine residue usually provided conformational flexibility to the adjacent peptide bonds (Fig. 1D). Thus, Glu-204 of VvOxyR2 is more likely than the Lys-203 residue to be responsible for the noncanonical conformation of the peptide bond.

Glu-204 is involved in the hypersensitivity of VvOxyR2 to H₂O₂

To examine the role of Glu-204, we generated a mutant V. vulnificus that harbors the VvOxyR2 (E204G) variant gene instead of wild-type VvOxyR2 and measured the minimum concentrations of H₂O₂ required for oxidation of VvOxyR2 in a bacterial cell. The bacterial strains expressing either the wild-type VvOxyR2 or the VvOxyR2 (E204G) variant were grown anaerobically and then were briefly exposed to various concentrations of H₂O₂. To facilitate analyses to determine the number of free thiols in the cellular VvOxyR2 proteins, the cells were treated with a 0.5-kDa alkylating agent, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS), that specifically reacts with a free thiol group. AMS linking would cause upshifts in the protein bands when visualized on SDS-polyacrylamide gel; the upshifts were proportional to the number of attached AMSs on the protein. To estimate the number of free thiols in VvOxyR2, the cells were treated with AMS, and all cellular proteins were then resolved on non-reducing SDS-PAGE and immunoblotted using anti-OxyR antibody to visualize the VvOxyR2 protein bands. The number of free thiols in VvOxyR2 cellular proteins was estimated by the upshift in protein bands on SDS-polyacrylamide gel. Upon oxidation, the molecular size of the resulting AMS-alkylated VvOxyR2 decreased by ~1 kDa because a disulfide bond that forms between the two redox-sensitive cysteines, Cys-206 and Cys-215, prevents alkylation of the two cysteine thiols (16). Oxidation of wild-type VvOxyR2 occurred within 30 s of exposure to 0.5 μM H₂O₂ (Fig. 2A, top), as reported previously (16). In contrast, the VvOxyR2 (E204G) variant was not oxidized until it was exposed to 5 μM H₂O₂ (Fig. 2B, top), suggesting that a Glu-204 to glycine mutation resulted in a significant increase in H₂O₂ level that can be sensed by VvOxyR2.

To assess the transcriptional activities of wild-type VvOxyR2 and the E204G variant, we next measured the transcript level of Vvprx2, a target gene of VvOxyR2 in V. vulnificus, using quantitative real-time PCR. The results demonstrated that Vvprx2 expression was activated by VvOxyR2 when exposed to exogenous H₂O₂ exceeding concentrations of 0.5 μM (Fig. 2A, bottom). In contrast, the VvOxyR2 (E204G) variant poorly activated Vvprx2 expression until exposed to 5 μM H₂O₂ (Fig. 2B, bottom). Collectively, these results indicate that Glu-204 of VvOxyR2 is a key residue for sensitivity to H₂O₂.

Crystal structures of the E204G variant

To ascertain the structural role of Glu-204, we determined three crystal structures of the VvOxyR2-RD (E204G) variant. Of these, the structure of the crystals grown under crystallization conditions similar to those of the wild-type protein exhibited the same space group (P3₁21) and packing constraints at a resolution of 1.55 Å, resulting in a SO₄-free high-resolution structure. Another structure was determined in a different space group (P3₁21) at a moderate resolution (2.3 Å). We additionally determined another crystal structure at 1.5 Å resolution, designated the SO₄-bound form, because it contains a sulfate ion near the peroxidatic cysteine residue. The overall structures of the variant were very similar to those of the wild-type protein except for the region around Gly-204 (Fig. 3, A and B).

In the SO₄-free high-resolution structure of the E204G variant, the peptide bond at the mutated region exhibited a dual
Crystal Structure of OxyR2

To analyze the structural role of Glu-204 of VvOxyR2, we generated a *V. vulnificus* variant strain harboring a VvOxyR2 (E204A) that would allow the role of the side chain to be excluded. This E204A variant VvOxyR2 was expected to have a peptide conformation similar to that of the wild-type VvOxyR2 having Glu-204, because only a glycine residue endows flexibility to the peptide bonds in proteins. As shown in Fig. 4A, this substitution exhibited an expression profile of Vpvr2 similar to the wild-type strain. Vpvr2 expression was induced by the mutant VvOxyR2 when treated with H$_2$O$_2$ exceeding concentrations of 0.5 μM, as observed with wild-type VvOxyR2 (Fig. 2A). This observation indicates that the peptide bond conformation of Glu-204 rather than the side chain of the Glu-204 is responsible for the hypersensitivity of VvOxyR2. We next excluded the role of the positive charge at Lys-203, which, like Glu-204, seems to be unique in VvOxyR2 (Fig. 1D, blue arrow). When the lysine residue was changed to a negatively charged Asp, a similar Vpvr2 expression profile was found (Fig. 4C).

We examined the structures of VvOxyR2 by focusing on the interactions involved in the peptides around Glu-204 or Gly-204. We noted that the carbonyl group of the peptide bond after Glu-204 or Gly-204 interacts directly with a chloride ion, which corresponds to the H$_2$O$_2$ molecule in the H$_2$O$_2$-bound PaOxyR C199D structure (Fig. 3, A and B) (9). More importantly, the peptide bond after Gly-204 exhibited a minor conformational duality, caused by the dual conformation of the peptide bond before Gly-204 in the SO$_4$-free high-resolution E204G structure that has the same packing constraints as the wild-type protein (Fig. 3C). The backbone carbonyl group of Gly-204 (or the carbonyl group of the peptide bond between Gly-204 and His-205) exhibited a 0.7-Å difference between the two conformations. This observation indicates motion or positional uncertainty of 0.7 Å in the carbonyl group involved in the binding of H$_2$O$_2$ near the peroxidatic cysteine residue (Figs. 3B and 5C). The conformational duality or flexibility in this carbonyl group contributes to the lower sensitivity of E204G variant VvOxyR2. In other words, the wild-type VvOxyR2 has a higher sensitivity to H$_2$O$_2$ due to the more rigid conformation resulting from employing Glu-204 instead of glycine with its intrinsic conformational flexibility.

**His-205 is important in sensing H$_2$O$_2$**

Due to the structural variation in the peptide bond after Glu-204 or between Glu-204 and His-205, the position of the side chain of His-205 was also affected by the conformation of the peptide bond (Fig. 5C). We thus investigated the role of the side chain of His-205. A *V. vulnificus* variant strain harboring the oxyR2 (H205A) mutant gene failed to express the target gene Vpvr2 at the indicated concentrations of H$_2$O$_2$, demonstrating the crucial role of His-205 in sensing H$_2$O$_2$ (Fig. 4B). Because His-205 interacts with an invariant water molecule (WT in Fig. 5, A and B), this conformational varia-

Figure 2. Effects of the Glu-204 mutation and transition to glycine on H$_2$O$_2$ sensing at low levels in VvOxyR2. The VvOxyR2 mutants containing plasmids expressing VvOxyR2 (A) or VvOxyR2 (E204G), in which Glu204 was mutated to glycine (B), were grown anaerobically to A$_{600}$ = 0.3 and exposed to various concentrations of H$_2$O$_2$, as indicated, for 30 s (top panels) or 3 min (bottom panels) before harvesting. The cells (top panels) were then precipitated with TCA and alkylated with fresh AMS buffer for 1 h at 37 °C. Alkylated VvOxyR2 proteins were resolved by non-reducing SDS-PAGE and immunoblotted using anti-VvOxyR2 antibody. The two redox states for each VvOxyR2 (reduced and oxidized) are shown with arrows. The negative control (NC) is the VvOxyR2 mutant expressing an empty vector. M, protein size marker. Total RNAs (bottom panels) were isolated, and the relative Vpvr2 transcript levels were estimated by quantitative real-time PCR analyses. The Vpvr2 mRNA level under anaerobic conditions (H$_2$O$_2$ of 0) was set to 1. Error bars from three independent experiments represent S.D.
tion at the side chain of His-205 also contributes to the sensitivity of VvOxyR2.

**VvOxyR2 has a second-order reaction rate constant 2 times higher than that of VvOxyR1**

To gain mechanistic insight into the relationship between the structural variations of VvOxyR2 and the sensitivity to H$_2$O$_2$, we measured the second-order reaction rate constants of VvOxyR2-RD and VvOxyR1-RD by analyzing competition kinetics with horseradish peroxidase-like peroxiredoxins (20). In the control, BSA and its 35 cysteine residues (one free cysteine residue) were employed. As shown in Fig. 6, both OxyR proteins exhibited constants of 10$^7$ M$^{-1}$ H$^{-1}$ 1 s$^{-1}$, and BSA displayed a negligible value in this assay. The rate constants for the OxyR RDs were similar to those for peroxiredoxins, indicating that the peroxidatic cysteines of OxyRs are oxidized to Cys-SOH by H$_2$O$_2$ as fast as occurs with peroxiredoxins. Noticeably, VvOxyR2-RD was about 2 times faster than VvOxyR1-RD, which may account for the higher sensitivity of VvOxyR2 than VvOxyR1. The faster reaction rate implies a lower acti-
vation energy of the reactions, and it would be more possible to perform the reaction even at a very low concentration of \( H_2O_2 \).

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\text{Figure 4. Effects of the Glu-204 and His-205 mutations on } H_2O_2 \text{ sensing at low levels in VvOxyR2. The VvoxyR2 mutants containing plasmids expressing VvOxyR2 (E204A) (A), VvOxyR2 (H205A) (B), and VvOxyR2 (K203D) (C) were grown anaerobically to an } A_{600} \text{ of 0.3 and exposed to various concentrations of } H_2O_2 \text{ as indicated, for 3 min. Total RNA isolation and quantitative real-time PCR analyses were performed as described previously. The } Vvpnx2 \text{ mRNA level under anaerobic conditions (} H_2O_2 = 0) \text{ was set as 1. All data are presented as mean ± S.D. (error bars); } n = 3.}
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\text{Figure 5. Structural variation of VvOxyR2 His-205 at the } H_2O_2 \text{ and water binding sites near the peroxidatic cysteine residue. The putative chloride ion in VvOxyR2 (A) and the } H_2O_2 \text{ molecule in } P. \text{ aeruginosa OxyR (B; PDB code 4X6G) are bound near the peroxidatic cysteine (C206) or the mutated aspartic acid (C199D) residue. The polar interactions involved in binding the chloride ion (gray ball) or } H_2O_2 \text{ (red ball and stick) are indicated by dotted lines. The bound water molecules (W1 and W2) are indicated by red balls. The } 2F_o - F_c \text{ (blue mesh) and } F_o - F_c \text{ (green mesh) omit maps of chloride ion and water molecules are contoured at 1.0 σ and 3.0 σ, respectively. C, two orthogonal views of the } SO_4^- \text{-free high-resolution structure of VvOxyR2 E204G variant. The alternative residues are presented by yellow or orange ball-and-stick representations. The difference points in dual conformation are indicated by red dihedral arrows. } t \text{ and } n \text{ letters in red indicate the typical conformation observed in the most OxyR proteins and the noncanonical conformation only observed in the wild-type VvoxyR2, respectively. * carbonyl atom between Glu-204 (or Gly-204) and His-205.}
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\text{Figure 6. Determination of the second-order rate constants of VvOxyR2-RD and VvOxyR1-RD. Reaction mixtures containing HRP and various concentrations of VvOxyR2-RD (●) or VvOxyR1-RD (○) (0 – 21.9 mM) were exposed to } H_2O_2. \text{ BSA (●) was used as a negative control. The ratio of HRP oxidation was determined by measuring the absorbance at 403 nm. } k_{VvOxyR2-RD} \text{ and } k_{VvOxyR1-RD} \text{ were determined from the slope of a plot of the equation, } (F/(1-F))k_{HRP}[HRP] \text{ versus [VvOxyR1-RD or VvOxyR2-RD]. The fitting lines were calculated by the least square methods, and the error bars reflect the S.D. of three independent experiments.}
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\text{Discussion}
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OxyR2 from \( V. \text{ vulnificus} \) has a distinctive amino acid sequence relative to other OxyRs and has a higher sensitivity to \( H_2O_2 \). However, the molecular underpinnings of the higher sensitivity of VvOxyR2 were unknown. To investigate the structural basis of the higher sensitivity of the protein, we determined the crystal structures of wild-type and an E204G variant of VvOxyR2-RD. In particular, we identified a VvOxyR2-specific residue, Glu-204, near the peroxidatic cysteine residue.
The plane of the peptide bond that occurs before Glu-204 was rotated compared with the other OxyR proteins that have a glycine residue at the Glu-204 site. Further analyses revealed that a VvOxyR2 variant harboring an E204G mutation exhibited decreased sensitivity to H$_2$O$_2$ in _V. vulnificus_ that was comparable with that of other OxyR proteins. Structural comparisons of the wild-type and the E204G variant VvOxyR2 provided a reasonable structural explanation for the higher sensitivity of VvOxyR2. The E204G mutation resulted in significant conformational flexibility in the region of Gly-204 and His-205. The carbonyl group of the peptide bond between His-205 and Gly-204 is involved in binding of a H$_2$O$_2$ molecule, and the imidazole ring of His-205 interacts with the invariant water molecule near the peroxidatic cysteine. Thus, our study suggests that the OxyR proteins regulate their sensitivity to H$_2$O$_2$ by altering the structural constraints of the peptide bond after Glu-204, which are involved in binding of H$_2$O$_2$ and a water molecule via the peptide bond with and the side chain of the His-205 residue.

What then is the mechanistic relationship between the structural variation of the OxyR proteins and the sensitivity to H$_2$O$_2$? The higher sensitivity could be accounted for by the higher affinity for H$_2$O$_2$ and the water molecules at the site near the peroxidatic cysteine residue. Unfortunately, we were not able to measure the binding constant of H$_2$O$_2$ due to the rapid reaction of H$_2$O$_2$ with the peroxidatic cysteine residue of the OxyR proteins. Instead, we measured the reaction rate constant for the decomposition of the H$_2$O$_2$ molecule, where H$_2$O$_2$ reacts. The results indicated that the structural alteration of VvOxyR2 may be related to the augmented reaction rate for the decomposition of the H$_2$O$_2$ molecule, where the structural alteration caused by Glu-204 provides a more favorable local environment for the reduction of the activation energy for the oxidation of the peroxidatic cysteine residue.

In many ways, OxyRs share the binding and reaction mechanisms with the peroxide-decomposing enzymes peroxiredoxins. OxyRs and peroxiredoxins have two invariant cysteine residues, which rapidly form a disulfide bond when exposed to H$_2$O$_2$. A H$_2$O$_2$ molecule is specifically bound near the peroxidatic cysteine (9, 21). Two-step oxidation mechanisms were proposed for both proteins. During the first step, disulfide formation in the proteins begins with the nucleophilic attack on the peroxide by the thiolate moiety in the peroxidatic cysteine residue, resulting in the Cys-SOH form (9, 12, 21). During the second step, the Cys-SOH form at the peroxidatic cysteine establishes a disulfide bond with the resolving cysteine in both proteins.

The second-order reaction rate constant for the disulfide bond formation of _E. coli_ OxyR, measured by differential cysteine modification followed by SDS-PAGE, was previously reported to be $\sim 10^7$ M$^{-1}$ s$^{-1}$ (22). However, in this study using competitive kinetics, the rate constants for VvOxyR2 and VvOxyR1 were on the order of $\sim 10^7$ M$^{-1}$ s$^{-1}$. This large discrepancy could be explained by differences under “Experimental procedures.” This study measured the second-order reaction constant for the first step ($k_1$) in oxidation mechanism (Fig. 7) because the competition experiment with horseradish peroxidase measured the consumption of H$_2$O$_2$. In the previous experiment, because they focused on the transition between the reduced form and the oxidized (or disulfide) form on SDS-polyacrylamide gels, the reaction rate constant for the overall reaction was measured. Thus, the previous values may reflect the rate constant ($k_j$) for the second step of disulfide bond formation, which is the rate-determining step in the two-step oxidation mechanism of OxyR (Fig. 7) (9, 12).

The higher second-order reaction rate constant also has implications for the high propensity for overoxidation of VvOxyR2. The Cys-SOH form at the peroxidatic cysteine has an alternative reaction pathway for overoxidation of the peroxidatic cysteine (Fig. 7). As proposed previously (9, 11), H$_2$O$_2$ could enter the H$_2$O$_2$ binding site even when the peroxidatic cysteine residue is oxidized to Cys-SOH, suggesting a similar reaction rate constant to $k_1$. Thus, our finding suggests that the
more sensitive OxyR with the higher $k_i$ value is more likely to enter the pathway leading to the overoxidation state instead of the oxidized state with the disulfide bond (Fig. 7). A similar explanation can also be applied to peroxiredoxins. In terms of gene regulation, the mechanistic common properties are important to maintain targeted sensitivity for optimal gene expression of peroxiredoxins by OxyR, depending on the cellular H$_2$O$_2$ level. Furthermore, the pathways leading to overoxidation of VvOxyR2 and VvPrx2 are also important in the fine regulation of gene expression.

This study shows that the thiol-based sensor OxyR regulates sensitivity to H$_2$O$_2$ by modulating the conformation of the residues required for binding of an H$_2$O$_2$ molecule. The VvOxyR2 strategy has implications for engineering other thiol-based sensor proteins with different sensitivities.

**Experimental procedures**

**Expression and purification of proteins**

VvOxyR2 (accession code: ADV87476) RD containing the amino acids 86–301 (VvOxyR2-RD) was amplified by PCR using the pair of primers described in Table 2. The PCR products were ligated into a His$_{6}$-tagged protein expression vector, pProEx-HTa (Invitrogen) (Table 3). The expression vector for VvOxyR2-RD (E204G) was constructed using overlapping PCR techniques (Table 2). His$_{6}$-tagged VvOxyR2-RD and VvOxyR2-RD (E204G) were expressed in *E. coli* BL21 (DE3) by the addition of 0.5 mM isopropyl-$\beta$-D-thiogalactopyranoside for 6 h at 30 °C. The expressed proteins, including the wild-type and mutant proteins, were purified using the same procedure described previously for P. aeruginosa OxyR RD (9). Briefly, the overexpressed VvOxyR2-RDs were purified by nickel-nitrilo-triacetic acid chromatography, anion-exchange chromatography (HiTrapQ, GE Healthcare), and size-exclusion chromatography (HiLoad 26/600 Superdex 200 pg, GE Healthcare). All purified proteins were concentrated to 30 mg/ml and stored at −80 °C until used for crystallization. Expression and purification of VvOxyR1-RD (accession code AIL71797, residues 80–299) used the same procedures as used for VvOxyR2-RD (Table 3).

**Structural determination**

Purified VvOxyR2-RD proteins (wild-type and E204G variants) were crystallized using the hanging-drop vapor diffusion method at 14 °C after mixing 1 µl of protein solution and 1.0 µl of precipitant solution. The wild-type VvOxyR2-RD protein was crystallized at 14 °C in a precipitation solution containing 0.2 M ammonium citrate (pH 6.5), 14% (w/v) PEG 3350, 2 mM tris(2-carboxyethyl)phosphine (TCEP), and 5 mM DTT. To obtain phase information, we soaked the crystals in 10 mM ZnCl$_2$-supplemented precipitation solution. The native and zinc-soaked crystals were flash-frozen in a nitrogen stream at −173 °C for X-ray diffraction using each crystallization solution supplemented with 20% (v/v) glycerol as a cryoprotectant. Native and single-wavelength anomalous diffraction (Zn-SAD) data were collected in Pohang Accelerator Laboratory beam lines. Phasing information and the initial model were obtained using a combination of molecular replacement and single-wavelength anomalous diffraction methods (MR-SAD) with the PHENIX Phaser-EP program and the Zn-SAD data set (17, 23). The final structure was refined with the PHENIX program against the native data set to a 1.8 Å resolution (24).

The E204G variant proteins of VvOxyR2-RD were also crystallized at 14 °C. The VvOxyR2-RD (E204G) protein was crystallized in three different conditions. One crystal for the SO$_4$-free, high-resolution structure of E204G variant was crystallized under the same conditions as wild-type crystals. Another crystal for the SO$_4$-free, high-resolution structure of E204G variant was crystallized in a solution containing 0.2 M sodium acetate, 0.1 M Tris-HCl (pH 9.0), 14% (w/v) PEG 4000, and 2 mM TCEP. The other crystal for the SO$_4$-bound structure was grown in a solution containing 0.2 M ammonium sulfate, 0.1 M sodium citrate (pH 5.6), 11% PEG 4000, and 2 mM TCEP. The E204G variant crystals were flash-frozen in a liquid nitrogen stream at −173 °C for X-ray diffraction using each crystallization solution supplemented with 20% (v/v) glycerol as a cryoprotectant. All structures of VvOxyR2 variants were determined by the molecular replacement method using the wild-type VvOxyR2-RD structure. The MOLREP program in the CCP4 package (25) was used for

**Table 2**

| Primer name | Oligonucleotide sequence (5’ → 3’) | Use |
|-------------|-----------------------------------|-----|
| For expression of VvOxyR RDs | | |
| OXYR2-RD-F | GGGCAAGACTCCATGGAACACTCTG07AG | Overexpression of VvOxyR2 RD |
| OXYR2-RD-R | GCTTCCAGCTGAGTGCTGAGTTGCGAGTGTCTCAAGT | Overexpression of VvOxyR1 RD |
| OXYR1-RD-F | GCTTCCAGTTACTCTGAGACCCTCAGC | |
| OXYR1-RD-R | GGTTCAGTTACTCTGAGACCCTCAGC | |
| For mutagenesis | | |
| OXYR2E204G-F | GTGATGTGATTTTTATGGAAAAAGGGCATTGTCTGACTGAAC | VvOxyR2 (E204G) variant |
| OXYR2E204G-R | GGGCAAGACTCCATGGAACACTCTG07AG | |
| OXYR2_E204A_F | GAGTCATGATTTTTATGGAAAAAGGGCATTGTCTGACTGAAC | VvOxyR2 (E204A) variant |
| OXYR2_E204A_R | GGTTCAGTTACTCTGAGACCCTCAGC | |
| OXYR2_H205A_F | GACACCGCGTGTTCAGTCAGACATGCCTTTTTCCAATAAAAATACTGAC | VvOxyR2 (H205A) variant |
| OXYR2_H205A_R | GGTTCAGTTACTCTGAGACCCTCAGC | |
| OXYR2K203D_F | GTCAGTCTCGAACATTCTCGCTCATCACTCTGAGTGTCCTCAAG | VvOxyR2 (K203D) variant |
| OXYR2K203D_R | GGTTCAGTTACTCTGAGACCCTCAGC | |
| For quantitative RT-PCR | | |
| PRX2QRT-F | GGTGCTTCTCGGTCTTCTCTCC | Quantification of the prx2 expression |
| PRX2QRT-R | TACCTGCTCCTGGCTCCTTCC | |

* The oligonucleotides were designed using the *V. vulnificus* MO6–24/O genomic sequence (GenBank™ accession number CP002469). Regions of oligonucleotides that are not complementary to the corresponding genes are underlined.
molecular replacement, and COOT (26) and PHENIX (24) were used to rebuild and refine the models. Crystallographic data and refinement statistics are shown in Table 1.

**Site-directed mutagenesis of VvoxyR2**

To determine VvOxyR2s mutant (E204G, E204A, H205A, and K203D) expression in *E. coli*; Table 2). (broad host-range vector, pJH0311, as template DNA) to create conjunction with the pDY1025 plasmid (Agilent Technologies) was used, according to previously described protocols (16). The complementary mutagenic primers, listed in Table 2, were used in conjunction with the pDY1025 plasmid (VvoxyR2 cloned into a broad host-range vector, pH0311, as template DNA) to create pBANG1414, pDY1025, pDY1607, and pDY1618 (for VvOxyR2 E204G, E204A, H205A, and K203D, respectively; Table 2). *E. coli* SM10 λ pir, tra (27) harboring pH0311, pDY1025, pBANG1414, pDY1025, pDY1607, pDY1608, and pDY1618 was used as a conjugal donor to the VvoxyR2 mutant (OH0703). The conjugation was conducted as described previously (16).

**In vivo alkylation of VvOxyR2 and Western blotting analysis**

The VvoxyR2 deletion mutant OH0703 with either pDY1025-expressing VvoxyR2 or pBANG1414-expressing VvoxyR2-E204G was used for Western blotting analysis of VvOxyR2 (Table 3), as described previously (16). Bacterial cells were grown anaerobically to an A550 of 0.3, aliquoted to an equal volume, and exposed to various concentrations of H2O2. To alkylate free thiols in the proteins with 0.5-kDa AMS (Invitrogen), the cells were immediately precipitated with 10% (w/v) ice-cold trichloroacetic acid, and the resulting pellets were dissolved in 50 μl of fresh AMS buffer (15 mM AMS, 1 M Tris (pH 8.0), 0.1% (w/v) SDS) (16). After incubation at 37 °C for 1 h, 3.5 μg of pelleted total protein was resolved on SD-SAGE under non-reducing conditions and immunoblotted with anti-VvOxyR2 polyclonal antibody, as described previously (15).

**RNA purification and transcript analysis**

Total cellular RNAs from the cultures grown anaerobically to an A550 of 0.3 were isolated using RNAprotect® bacteria reagent and the RNEasy® minikit (all from Qiagen). The cells were exposed to given concentrations of H2O2 for 30 s or for 3 min in culture media and then harvested. cDNA was synthesized using the iScript™ cDNA synthesis kit (Bio-Rad), and the Vvpnx2 expression level was measured by quantitative RT-PCR amplification of the cDNA using the Chromo 4 real-time PCR detection system (Bio-Rad). The primer pair sequences are 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 (28). Quantitative RT-PCR data are presented as mean ± S.D. of three independent experiments.

**Competitive kinetics with horseradish peroxidase (HRP)**

The purified proteins VvOxyR2-RD and VvOxyR1-RD were reduced by incubation with a buffer (20 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1 mM DTT) for 60 min at room temperature. Residual DTT was removed by ultrafiltration using 10-kDa Amicon Ultra centrifugal filter devices (Merck Millipore, Darmstadt, Germany). The reaction mixtures containing 5.84 μM HRP (Sigma) and various concentrations (0–21.9 μM) of the reduced VvOxyR2-RD or VvOxyR1-RD were treated with 4 μM H2O2 at room temperature in reaction buffer containing 20 mM Tris-Cl (pH 8.0), 150 mM NaCl, and 1 mM EDTA. The concentration of HRP was calculated by absorbance measurement at 403 nm (ε403 = 1.02 × 10^5 M⁻¹ cm⁻¹), and the concentrations of other proteins were determined spectrophotometrically based on the molar extinction coefficients at 280 nm. The competitive kinetics of VvOxyR1 and VvOxyR2-RD were determined using a similar procedure, as described previously (20, 29). In brief, the ratio of inhibition of horseradish peroxidase oxidation (F(1 − F)) was measured at 403 nm using a Tecan Infinite M200 reader (Tecan, Mannedorf, Switzerland). The second-order rate constants (k) of VvOxyR1-RD and VvOxyR2-RD were determined from the slope of the plot of the equation, (F(1 − F))kHRP/[VvOxyR1-RD or VvOxyR2-RD] (kHRP = 1.7 × 10^7 M⁻¹ s⁻¹).

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Crystal Structure of OxyR2

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