Structural snapshots of OxyR reveal the peroxidatic mechanism of H$_2$O$_2$ sensing

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Hydrogen peroxide (H$_2$O$_2$) is a strong oxidant capable of oxidizing cysteinytl thiolates, yet only a few cysteine-containing proteins have exceptional reactivity toward H$_2$O$_2$. One such example is the prokaryotic transcription factor OxyR, which controls the antioxidant response in bacteria, and which specifically and rapidly reduces H$_2$O$_2$. In this study, we present crystallographic evidence for the H$_2$O$_2$-sensing mechanism and H$_2$O$_2$-dependent structural transition of Corynebacterium glutamicum OxyR by capturing the reduced and H$_2$O$_2$-bound structures of a serine mutant of the peroxidase cysteine, and the full-length crystal structure of disulfide-bonded oxidized OxyR. In the H$_2$O$_2$-bound structure, we pinpoint the key residues for the peroxidatic reduction of H$_2$O$_2$, and relate this to mutational assays showing that the conserved active-site residues T107 and R278 are critical for effective H$_2$O$_2$ reduction. Furthermore, we propose an allosteric mode of structural change, whereby a localized conformational change arising from H$_2$O$_2$-induced intramolecular disulfide formation drives a structural shift at the dimerization interface of OxyR, leading to overall changes in quaternary structure and an altered DNA-binding topology and affinity at the catalase promoter region. This study provides molecular insights into the overall OxyR transcription mechanism regulated by H$_2$O$_2$.

transcription factor | hydrogen peroxide sensor | redox regulation | X-ray structure

H$_2$O$_2$ is a strong oxidant that mainly targets metal centers and protein thiol groups (1, 4). However, within thios, there is a great gap between the oxidation rate of most thiol groups (∼10–100 M$^{-1}$s$^{-1}$) and the oxidation rate of specialized protein thiols (≥10$^3$ M$^{-1}$s$^{-1}$), meaning that only a handful of proteins are relevant for controlling the intracellular H$_2$O$_2$ levels. These proteins have additional structural features, which lower the otherwise high-energy activation barrier of H$_2$O$_2$ reduction, speeding up the otherwise slow reaction (4). Such features have been studied in detail for peroxidiredoxs (Pdrs), where a Thr/Cys/Arg triad is mainly responsible for the peroxidatic activity (5, 6). Both the Thr and the Arg favor the reduction of H$_2$O$_2$ by stabilizing the transition state and by polarizing the O−O peroxyl bond (5). For other cases, as in the sulfur-containing glutathione peroxidase family, a Cys/Asn/Gln/Thr tetrad is responsible for the peroxidatic activity, but the details of the H$_2$O$_2$ reduction mechanism have not yet been uncovered (7). In general, structural factors that govern thiol-based H$_2$O$_2$ sensing and catalysis of H$_2$O$_2$ reduction are still unclear.

One protein with exceptionally high reactivity toward H$_2$O$_2$ is the prokaryotic transcription factor OxyR, a member of the LysR-type transcriptional regulator (LTTR) family. LTTRs contain two domains: an N-terminal DNA-binding domain (DBD) and a C-terminal regulatory domain (RD), responsible for sensing specific metabolites (H$_2$O$_2$ in the case of OxyR). OxyR uses its H$_2$O$_2$-reactive peroxidatic cysteine (C$_{\mathrm{P}}$) of its RD domain to sense increased H$_2$O$_2$ concentrations, reacting with H$_2$O$_2$ at a rate of ∼10$^3$ M$^{-1}$s$^{-1}$ (8) and, upon intramolecular resolution of the sulfenylated C$_{\mathrm{P}}$ with the resolving cysteine (C$_{\mathrm{R}}$), transudes this oxidative signal into the transcription of H$_2$O$_2$-detoxifying genes, such as catalase, and iron-chelating proteins (9–12). For many virulent bacteria, OxyR is an important antioxidant defense regulator, as OxyR knockouts are sensitive to oxidative stress and exhibit attenuated virulence (13–19). The sensitivity of OxyR toward H$_2$O$_2$ has been exploited to create the HyPer probe, a genetically encoded

Significance

The bacterial transcription factor OxyR is a model example of a highly sensitive and specific hydrogen peroxide (H$_2$O$_2$) sensor. H$_2$O$_2$ reduction by its active-site cysteine triggers protein structural changes leading to an increased transcription of antioxidant genes. By solving the crystal structures of full-length OxyR in both reduced and oxidized states, we provide molecular insight into these structural changes. We also present a H$_2$O$_2$-bound structure with a threonine activating the peroxidase, and argue that this H$_2$O$_2$-bound structure may be catalytically more relevant than that seen previously in the study of a sulfenic acid-mimic mutant of the active-site cysteine. Finally, we discuss the commonalities and differences between the peroxidatic mechanisms of peroxidiredoxs and OxyR.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 6G1D, 6G4R, and 6G18).

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fluorescent biosensor that dynamically monitors physiological fluctuations in cellular H$_2$O$_2$ concentrations (20, 21).

The first published crystal structures of the oxidized and reduced states of the RD of Ec-OxyR provided a model in which a rate-limiting conformational change follows C-C-C disulfide formation (8, 22). To date, crystal structures of the OxyR RD from *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio vulnificus*, *Neisseria meningitidis*, and *Porphyromonas gingivalis* have been published, displaying a conserved homodimeric arrangement of RD subunits (22–26). So far, the molecular evidence for H$_2$O$_2$ binding and catalysis at the peroxidatic active site of OxyR derives from crystallographic and biochemical studies of *P. aeruginosa* OxyR (Pa-OxyR). A crystal structure of full-length Pa-OxyR, with the C$_2$ mutated to Asp, a sulfanilylated cysteine mimic, has been solved with H$_2$O$_2$ bound in the active site, and mutational studies highlighted a role of the active-site residue T100 (Pa-OxyR numbering) for H$_2$O$_2$ reduction (23).

In this study, we focus on the catalytic and regulatory mechanisms of a noncanonical OxyR from the actinomycete *Corynebacterium glutamicum* (C$_g$-OxyR), which utilizes a repressor mechanism toward catalase transcription, differing from the typical *E. coli* OxyR model, which uses an activator mechanism (10, 27, 28). Here, we present the reduced, H$_2$O$_2$-bound, and intramolecular disulfide-bonded crystal structures of C$_g$-OxyR, and find an altered H$_2$O$_2$ binding configuration with respect to the Pa-OxyR findings (23). By combining structural data with H$_2$O$_2$ consumption assays on selected site-directed mutants of OxyR, we show the critical residues for H$_2$O$_2$ reduction. The structural changes following disulfide formation in C$_g$-OxyR point toward an allosteric mechanism that regulates the RD homodimeric interface and translates into conformational changes in the OxyR tetrameric assembly. Finally, we explore how C$_g$-OxyR acts as a H$_2$O$_2$-sensing transcriptional regulator in vivo and discuss the implications of its mode of action.

**Results**

**Full-Length C$_g$-OxyR Structure Shows a Tetrameric Configuration Differing from Previously Published Structures.** The crystal structure of full-length C$_g$-OxyR$_{C206S}$ (a C$_2$ mutant, C206S, which functionally is redox-insensitive) was solved to a resolution of 2 Å in space group P2$_1$ (Table 1). The asymmetric unit contains a homotetramer composed of a dimer of dimers, wherein an intradimer interface is formed between the RDs of each respective protomer, and an interdimer interface is formed between the DBDs from each dimer (Fig. 1B). The tetrameric oligomeric state of reduced OxyR was supported by high-resolution size-exclusion chromatography (SI Appendix, Fig. S1A). The overall structural fold of the RD is common to that of the LLTR family, with an N-terminal domain comprised of β-strands sandwiched between two α-helices (subdomain 1), a bundle of β-strands and α-helices (subdomain 2), and a C-terminal α-helix and β-strand traversing both domains (Fig. 1C). The tetrameric conformation of C$_g$-OxyR$_{C206S}$ differs noticeably from the tetrameric structure of Pa-OxyR (Fig. 1A) (PDB ID code 4XG6) (23) and the putative *P. aeruginosa* LLTR PA01 (PDB ID code 3FZV). Although the interdimeric interface of the DBDs itself is conserved among the three structures, the tetrameric configuration between the three structures derives from a difference in the hinge bending angle between the DBD and RD subunits. When considering the RD homodimers of Pa-OxyR, an asymmetry in the angle between the RD and the DBD of each subunit of the dimer is apparent. Whereas the DBD of one subunit hinges at an ~55–60° acute angle relative to the connecting RD, the DBD of the other subunit hinges at an ~100° obtuse angle relative to the connecting RD. This combination of acute and obtuse angle hinging of the homodimer is reflected in the paring homodimer, which constitutes the full tetramer, therefore resulting in a “slanted” tetrameric assembly. The subunits of the C$_g$-OxyR$_{C206S}$ structure, on the other hand, all exhibit similarly acute hinging angles between the DBD and RD of each subunit, all with hinge angles in the range of 40–50°. This results in a relatively more symmetric assembly of subunits within the C$_g$-OxyR$_{C206S}$ tetramer. The hinging angle was determined by the intersect between the midpoint and the C terminus of DBDα4, and the C terminus of DBDε4 and N terminus of RDβ1 (see Fig. 1C for secondary structure annotation), giving a hypoteneuse between the midpoint of DBDα4 and the N terminus of RDβ1.

**Conserved Architecture of the OxyR Active-Site Pocket.** In the crystal structure of C$_g$-OxyR$_{C206S}$, a sulfate molecule occupies the peroxidatic active-site pocket. This sulfate engages in hydrogen bonds with the side-chain hydroxyls of T107 and S206 (C$_2$ equivalent) and the side-chain amine of H205 (Fig. 2A). A sulfate molecule is also present in the crystal structure of *V. vulnificus* OxyR2 (Vv-OxyR2; PDB ID codes 5B7D and 5B7H), binding at an identical location and with the same hydrogen bond interactions (Fig. 2A) (25). In other OxyR structures, there is a conserved network of three water molecules within the active-site pocket instead of a sulfate molecule (PDB ID codes 3T22, 3TV9, and 4Y0M) (Fig. 2A) (22–26). This water pattern is conserved among these structures due to hydrogen bond interactions with side-chains of four conserved residues—T107, T136, H205, and C206—in C$_g$-OxyR (in *N. meningitidis* OxyR, H205 is substituted by Asn). Just outside of the active-site pocket, an additional conserved water molecule binds to both the side-chains of the C$_2$ (Ser in C$_2$-mutant) and R278, the latter of which also contributes hydrogen bonds via its guanidinium group to the oxygens of the side-chain hydroxyl and backbone carboxyl of T107, an interaction that may play a role in stabilizing the position of T107 within the active-site architecture (Fig. 2A). Conservation of these active-site residues is demonstrated by a multiple sequence alignment of 67 OxyRs from different families (SI Appendix, Fig. S2). In particular, the C$_2$ and the R278 are universally conserved (SI Appendix, Fig. S2), while T107 is present in 97% of the sequences (substituted by a serine in the remaining 3%).

### Crystallographic and Kinetic Evidence for H$_2$O$_2$ Binding and Reduction.

To better understand the role of these conserved residues in

| Table 1. X-ray data collection and refinement statistics | OxyR$_{C206S}$ (6G1D) | OxyR$_{H2O2}$ (6G4R) | OxyR$_{SG}$ (6G1B) |
|---------------------------------------------------------|------------------------|----------------------|-------------------|
| **Data collection**                                      |                        |                      |                   |
| Space group                                             | P2$_1$                 | P2$_1$               | C2                |
| Cell dimensions                                        |                        |                      |                   |
| a, b, c (Å)                                            | 74.4, 63.5             | 71.2, 57.0           | 161.3, 46.2       |
| β (°)                                                   | 97.8                   | 98.0                 | 121.9             |
| Resolution (Å)                                         | 49.23–1.99             | 152.67–2.62          | 46.07–2.28        |
| Rmerge                                                 | (2.03–1.99)*           | (2.66–2.62)*         | (2.36–2.28)*      |
| Completeness (%)                                        | 99 (85.1)              | 99.8 (95.1)          | 96.6 (91.4)       |
| Redundancy                                              | 7 (6.1)                | 6.5 (6.5)            | 5.1 (4.7)         |
| **Refinement**                                          |                        |                      |                   |
| Resolution (Å)                                         | 44.86–1.99             | 76.34–2.62           | 46.06–2.28        |
| No. reflections                                        | 98,622 (9050)          | 36,733 (3460)        | 30,760 (2245)     |
| Rmerge/Rfree                                           | 0.200/0.221            | 0.235/0.286          | 0.196/0.230       |
| No. atoms                                               | 10,408                 | 9,338                | 5,192             |
| Protein                                                | 9.670                  | 9.218                | 4.883             |
| **Bond lengths (Å)**                                    |                        |                      |                   |
| Ligand/ion                                              | 70                     | 15                   | 28                |
| Water                                                   | 668                    | 405                  | 281               |
| Rmsd                                                    | 50.0                   | 71.2                 | 52.2              |
| **Bond angles (°)**                                     | 1.7                    | 0.6                  | 1.8               |

*Values in parentheses are for highest-resolution shell.*
H$_2$O$_2$ binding and reduction at the molecular level, we soaked crystals of the redox-insensitive Cg-OxyR$_{C206S}$ with H$_2$O$_2$ to gain a peroxide-bound structure. Exposure of crystals of Cg-OxyR$_{C206S}$ to 600 mM H$_2$O$_2$ for 90 min enabled determination of a crystal structure of H$_2$O$_2$-bound Cg-OxyR$_{C206S}$ (hereafter referred to as Cg-OxyR$_{C206S}$H$_2$O$_2$) at a resolution of 2.6 Å in space group P$_2_1$ (Table 1). Cg-OxyR$_{C206S}$H$_2$O$_2$ retains an overall tetrameric configuration close to that of Cg-OxyR$_{C206S}$, with some minor subunit displacement. In all four respective active-site pockets of the Cg-OxyR$_{H2O2}$ tetramer, the sulfate molecule is no longer present, and in two of the four pockets a molecule of H$_2$O$_2$ and a water molecule occupy a position equivalent to the sulfate (Fig. 2B). The structural architecture of the remaining two active-site pockets is disrupted by unfolding of the $\alpha_3$ helix of the RD (RD$_{\alpha_3}$), where the C$_P$ and C$_R$ reside, into a loop. In both of the

![Fig. 1. Cg-OxyR crystal structure shows a more symmetric subunit arrangement compared with the asymmetric relation between the RD and DBD homodimers of Pa-OxyR. (A) Crystal structure of tetrameric Pa-OxyR C199D [PDB ID code 4X6G (23)]. (B) Crystal structure of tetrameric Cg-OxyR$_{C206S}$. (C) Overview of the Cg-OxyR$_{C206S}$ monomeric subunit, with the DBD and the RD highlighted.](image)

![Fig. 2. Comparison of Cg-OxyR active-site pocket with previously published structures. (A) OxyR structures show conservation of the active-site region. (A) OxyR structures show conservation of the active-site region.](image)
peroxide-bound pockets one oxygen of H$_2$O$_2$ (O$_A$) accepts a hydrogen bond from the side-chain hydroxyl of T107 and the backbone amide of S206, and the other peroxide oxygen (O$_B$) is bridged to the side-chain hydroxyl of T136 by a water molecule (Fig. 2B). The location of the H$_2$O$_2$ moiety in the active-site pocket is different to the one found in the C199D Pa-OxyR structure: in this structure, the O$_A$ peroxide oxygen establishes hydrogen bond contacts with the side-chain carboxyl of D199 and the side-chain hydroxyl of T129 (T136 in Cg-OxyR) (Fig. 2B) (23). The side-chain conformation of S206 is variable between the two H$_2$O$_2$-bound pockets, and in one pocket (hereafter referred to as “pocket A”) is directed away from the H$_2$O$_2$-containing active site, whereas in the other pocket (now referred to as “pocket B”) is directed toward H$_2$O$_2$ and would be within distance for a potential thiolate nucelophilic attack with the native cysteines (Fig. 3A).

To explore the importance of the conserved active-site residues T107, T136, H205, C206, and R278 in peroxide reduction, single-residue variants (T107V, T136V, H205A, C206S, R278Q) were constructed, and H$_2$O$_2$ consumption was monitored in function of time using the ferrous oxidation of xylene orange (FOX assay), which detects the H$_2$O$_2$ concentration in a given solution. While WT Cg-OxyR consumes the majority of H$_2$O$_2$ within 10 s, T107V and C206S variants are catalytically dead and only reduce minor amounts of H$_2$O$_2$ (Fig. 3B). The peroxidatic activity of the R278Q mutant is clearly affected, but not as dramatically as the T107V or C206S variant, which detects the H$_2$O$_2$ concentration compared with WT Cg-OxyR (SI Appendix, Fig. S3). Taken together, the combination of structural and kinetic data points to a hierarchical contribution of four active-site residues for H$_2$O$_2$ binding and reduction in the following order of importance: C206, T107, R278, and T136.

**Disulfide Bond Formation Reorganizes the OxyR Tetrameric Assembly.**

The crystal structure of the intramolecular disulfide-bonded form of Cg-OxyR (Cg-OxyRSS) was solved to a resolution of 2.3 Å in space group C2 (Table 1). The asymmetric unit cell contains two protomers of OxyR forming a RD-interfacing homodimer, which is one-half of the full tetramer related by crystallographic symmetry (Fig. 4A). The tetrameric oligomeric state of Cg-OxyRSS was confirmed by high-resolution size-exclusion chromatography, with some octameric subpopulation (SI Appendix, Fig. S4A). As with Cg-OxyR$_{\text{C206S}}$, the crystallographic tetramer of Cg-OxyRSS is a dimer of RD-interfaced homodimers with interhomodimer interactions predominating through respective DBD interfaces, although its overall tetrameric conformation differs significantly from that of Cg-OxyR$_{\text{C206S}}$ (Fig. 4A). The different oligomeric configurations adopted by Cg-OxyR$_{\text{C206S}}$ and Cg-OxyRSS arise predominantly from an asymmetric conformation differences in the DBD to the RD, resulting in a more square-like conformation for Cg-OxyRSS compared with the more rhomboid shape of Cg-OxyR$_{\text{C206S}}$ with a shortening of the distance between DBDs of an RD homodimer from ~117 Å in Cg-OxyR$_{\text{C206S}}$ to ~96 Å in Cg-OxyRSS (Fig. 4A). Despite this significant oligomeric rearrangement, the interprotomer DBD interfaces are essentially conserved between the oxidized and reduced forms of OxyR (SI Appendix, Fig. S4B).

In both protein chains of the asymmetric unit, the C$_\text{R}$ (C206) and C$_\text{B}$ (C215) engage in a C$_\text{R}$-C$_\text{B}$ intramolecular disulfide. This disulfide formation is facilitated by unfolding of the RDα3 helix, displacing the C$_\text{R}$ by 9.8 Å and C$_\text{B}$ by 7.4 Å (C$_\text{R}$-C$_\text{B}$), and resulting in the formation of a short $\beta$-strand from C206 to H208 (Fig. 4B). Of note, the unfolded conformation of the RDα3 helix is almost identical to that observed in Cg-OxyR$_{\text{H2O2}}$ (Fig. 4C). These local conformational changes induce a subtle repacking of the hydrophobic core proximal to the peroxidatic site, causing allosteric structural changes at the RD homodimer interface, which translates into a cumulative 20° sliding rotation of the RD homodimer protomers. For a more in-depth discussion of the structural factors governing the localized conformational changes upon oxidation of Cg-OxyR, see SI Appendix.

**H$_2$O$_2$ Induces Rapid Intersubunit Rotation.**

The single Trp of Cg-OxyR, W258, is located at the RD dimerization interface and shows a side-chain flip upon Cg-OxyR oxidation (Fig. 5A), which causes a decrease in the Trp intrinsic fluorescence (Fig. 5B). This fluorescence decrease can be reverted upon addition of the reducing agent DTT (Fig. 5B). Motivated by this observation, we used the fluorescent read-out for a stopped-flow kinetic study. Upon H$_2$O$_2$ exposure, a rapid exponential fluorescence decrease was observed for WT Cg-OxyR RD (Fig. 5C). The C206S variant, which is peroxidatically inert, has almost no fluorescence decrease, while the C215S variant, which is peroxidatically active, shows a fluorescence decrease but lower than for WT Cg-OxyR (Fig. 5C). This further supports a mechanism in which C$_\text{R}$-C$_\text{B}$ disulfide formation is required to lock the RD intersubunit.
rotation in Cg-OxyR, leading to a side-chain flip of W258. The progress curve for WT Cg-OxyR fits with an equation for a single exponential decay (Fig. 5D), and the observed rate constants of the progress curves correlate with the H$_2$O$_2$ concentration in a hyperbolic manner (Fig. 5D). Fitting the data to a hyperbolic equation \( k_{obs} = \left(\frac{E_0 k_{cat} [H_2O_2]}{K_M + [H_2O_2]}\right) \) gives a \( k_{cat} \) of 105.7 s$^{-1}$ and a \( K_M \) of 2.56 mM, resulting in a \( k_{cat}/K_M \) of 4.2 $\times$ 10$^4$ M$^{-1}$s$^{-1}$. Using the same approach, we also evaluated the kinetic impact on the T136V and H205A variants. These mutants did not show a single exponential fluorescence decrease upon H$_2$O$_2$ addition, but instead gave a single exponential increase in fluorescence, which, for the H205A variant, was preceded by a rapid decrease in fluorescence (SI Appendix, Fig. S5). It is possible that these mutations slightly reposition W258, which upon H$_2$O$_2$ addition affects the overall protein intrinsic fluorescence. For both H205A and T136V mutants, the observed rate constants of the fluorescence increase are linearly proportional to the H$_2$O$_2$ concentration (SI Appendix, Fig. S5). From the slope of the linear curve, a second-order rate constant of (1.06 $\pm$ 0.1) $\times$ 10$^7$ M$^{-1}$s$^{-1}$ for H205A and (5.18 $\pm$ 0.19) $\times$ 10$^7$ M$^{-1}$s$^{-1}$ for T136V (SI Appendix, Fig. S5), which is in good agreement with the results obtained from the FOX assay (Fig. 3B). For the R278Q variant, we were unable to determine the second-order rate constant via this approach. As an alternative, we used the FOX assay with a fixed H$_2$O$_2$ concentration while varying the concentration of R278Q Cg-OxyR. The H$_2$O$_2$ consumption profile followed a single exponential decrease, and the observed rate constant was linearly proportional to the concentration of R278Q Cg-OxyR (SI Appendix, Fig. S5), yielding a second-order rate constant of (1.79 $\pm$ 0.12) $\times$ 10$^7$ M$^{-1}$s$^{-1}$. A summary of the rate constants can be found in Table 2. Overall, Cg-OxyR exhibits a rapid conformational change upon oxidation, which is slowed by replacement of the conserved active-site residues. Disulfide-Bonded Cg-OxyR Shifts Its DNA-Binding Pattern at the Catalase Regulatory Region. Contrary to the conventional E. coli model of OxyR as a transcriptional activator, the OxyRs of Corynebacterium diphtheriae and C. glutamicum are thought to act as transcriptional repressors (27, 29). It has been demonstrated that the oxidized, intramolecular disulfide-bonded form of Cg-OxyR is less specific in its binding to the four promoter regions, katA, dps, fin, and cydA relative to reduced Cg-OxyR, and that in vivo bacterial exposure to H$_2$O$_2$ leads to induction of katA and dps, but not fin and cydA (27). We aimed to assess the
relationship between the oxidation state of Cg-OxyR and its ability to bind to the catalase (katA) operator/promoter region. Using EMSA, we found that both oxidized and reduced Cg-OxyR bind to the catalase operator/promoter region, and this with no apparent difference in the electrophoretic mobility of the Cg-OxyR-DNA complex (SI Appendix, Fig. S6A). We found that only the DBDs bind to the katA regulatory region, because a Cg-OxyR RD-only variant does not show any interaction (SI Appendix, Fig. S6B). Next, to determine the exact Cg-OxyR binding region, we performed DNase I footprinting experiments, and observed that reduced Cg-OxyR protects a section corresponding to approximately four consecutive helical turns, which includes the −10 promoter element and the transcription start point (Fig. 6A). On the other hand, disulfide-bonded Cg-OxyR retained binding to the −10 promoter element, but the last two helical turns were no longer protected from the DNase I treatment (Fig. 6A). The Cg-OxyR_C206S variant protected the same katA regulatory region as reduced WT Cg-OxyR, whereas reduced Cg mutant (Cg-OxyR_C215S) displayed slightly less protection of the binding region in comparison with reduced WT or Cg-OxyR_C206S (Fig. 6A). Treatment of Cg-OxyR_C215S with H2O2 causes a uniform decrease of protection on the whole binding region, whereas protection by H2O2-treated Cg-OxyR_C206S was unaffected (Fig. 6A).

To determine the affinity of the Cg-OxyR variants toward the protected region of the katA promoter, we measured binding to a 6-carboxyfluorescein (6-FAM)-labeled katA promoter oligonucleotide by fluorescence polarization, whereby binding of OxyR to the oligonucleotide increases the fluorescence polarization. Reduced WT Cg-OxyR, and reduced and H2O2-treated Cg-OxyR_C206S gave similar affinities (∼40 nM) for the oligonucleotide and bound with strong positive cooperativity (h = 2.6–2.8) (Fig. 6B), whereas oxidized WT Cg-OxyR had a fourfold lower binding affinity (∼160 nM) with almost no cooperativity (h = 1.2) (Fig. 6B). Isothermal titration calorimetry of the interaction between Cg-OxyR and the katA promoter showed that binding of the katA promoter to reduced Cg-OxyR occurs with a higher affinity than that observed for binding to oxidized Cg-OxyR, and displays an initial exothermic binding step not observed for oxidized Cg-OxyR (SI Appendix, Fig. S6C).

Next, to identify the nitrogenous bases of the katA promoter that are directly involved in the binding to reduced and/or oxidized Cg-OxyR, missing contact probing experiments were performed. These experiments involve addition of Cg-OxyR to sparingly modified depurinated/depyrimidinated DNA (on average one modification per molecule), followed by separation of the free DNA and protein-bound forms on an acrylamide gel, cleavage of the DNA backbone at the modified positions, and separation of the reaction products by gel electrophoresis under denaturing conditions. If a base is essential to Cg-OxyR binding, DNA molecules missing this base will be underrepresented in the bound DNA form and overrepresented in the free DNA form (30). Initially, the missing contact probing was tested with oxidized WT Cg-OxyR and with the redox-insensitive C206S variant, Cg-OxyR_C206S. However, it was observed that the complex between Cg-OxyR_C206S and DNA gave a totally different electrophoretic mobility pattern on the acrylamide gel compared with the WT Cg-OxyR-DNA

Table 2. Second-order rate constants of OxyR reaction with H2O2 (pH 7.4, 25 °C)

| OxyR variant | k (M⁻¹s⁻¹) |
|--------------|------------|
| WT           | 4.20 × 10⁴  |
| H205A        | 1.06 × 10⁴  |
| T136V        | 5.18 × 10²  |
| R278Q        | 1.79 × 10²  |
complex (SI Appendix, Fig. S6D), and so for this reason we decided not to use the Cg-OxyR C206S variant for this experiment. For reduced WT Cg-OxyR, we found two binding regions, each 11- to 13-nucleotides long and separated by 6 nucleotides (SI Appendix, Fig. S7). Both reduced and oxidized Cg-OxyR specifically bound the upstream binding region, which contains the −10 promoter element, and is immediately upstream of the transcription start point, but the oxidized form of Cg-OxyR was no longer able to specifically interact with the downstream binding region (SI Appendix, Fig. S7).

**Cg-OxyR Is a Repressive Gatekeeper of Catalase Expression.** Because Cg-OxyR binds to the catalase regulatory region in a manner dependent on its oxidation state, we explored the *C. glutamicum* response to H$_2$O$_2$ stress by evaluating the regulation of catalase transcription. Under basal conditions, a ΔoxyR strain of *C. glutamicum* displayed a massive increase of the *katA* mRNA levels, ∼20-fold higher than in the WT strain, indicating that Cg-OxyR represses *katA* transcription (Fig. 7A and SI Appendix, Table S2). In agreement with this, complementation of ΔoxyR with overexpressed WT Cg-OxyR (which displays an ∼20-fold increase of the oxyR mRNA level) (SI Appendix, Table S3) led to a great decrease in *katA* expression (as expected), equivalent to the one described for the WT strain (Fig. 7A and SI Appendix, Table S2). In contrast, complementation with C206S or C215S oxyR increased *katA* expression compared with the WT strain (∼two- to fourfold) (Fig. 7A and SI Appendix, Table S2).

We also evaluated the fluctuations of *katA* mRNA levels in response to externally applied H$_2$O$_2$ for the same strains (Fig. 7B and SI Appendix, Table S2). For reduced WT oxyR, we found two binding regions, each 11- to 13-nucleotides long and separated by 6 nucleotides (SI Appendix, Fig. S7). Both reduced and oxidized Cg-OxyR specifically bound the upstream binding region, which contains the −10 promoter element, and is immediately upstream of the transcription start point, but the oxidized form of Cg-OxyR was no longer able to specifically interact with the downstream binding region (SI Appendix, Fig. S7).

**Discussion**

The generation of reactive oxidants is key for the defense of the host against pathogenic invasion. As the frontline sensor and coordinator of the bacterial response to the oxidant H$_2$O$_2$, OxyR drives the survival of many pathogenic organisms (9–12, 14–19). Its efficacy of defensive coordination depends on the ability to sense intracellular H$_2$O$_2$ before it reaches a concentration that inhibits bacterial growth (1, 3).

Here we show that the H$_2$O$_2$-sensing capacity of OxyR relies mainly on three conserved residues (T107/C206/R278 in Cg-OxyR), and that water molecules in the active-site pocket likely play a supplementary role in proton transfer. We find a conserved water placement among the peroxidatic pockets of multiple OxyRs, and a close match between the location of conserved waters and the respective positions of the O$_{A1}$ and O$_{B2}$ in the H$_2$O$_2$-bound structure (5). Because the C$_P$ of both OxyR and Prdx facilitate peroxide reduction at rates several orders-of-magnitude greater than that for a typical protein thiol, key insights can be gained from comparison of the contribution of the respective residues in active-site environments (Fig. 8). In both cases, hydrogen-bonding interactions are predominantly formed at O$_{A1}$ over O$_{B2}$ and whereas the C$_P$ thiolate
of Prdx is stabilized by hydrogen bond donation from an arginine guanidinium group, we propose that in OxyR this hydrogen bond instead comes from a water molecule bridging R278 and Q210. For both Prdx and OxyR, a conserved Thr plays a critical role in providing a hydrogen bond to the peroxide O₂⁻ (Fig. 8; see SI Appendix for further discussion of the molecular architecture of H₂O₂ binding in Cg-OxyR). Our study shows the essential nature of T107 hydroxyl group for H₂O₂ reduction by Cg-OxyR, a finding supported by the previous observation that a T100V mutation in Pa-OxyR induces cellular H₂O₂ hypersensitivity (23). In Cg-OxyR, mutating R278 also impairs H₂O₂ reduction, and we postulate that R278 scaffolds the active-site architecture by correctly positioning T107 for its catalytic role (Fig. 8). Based on these results, we propose that OxyR possess a core triad (T/C/R) for H₂O₂ reduction.

Following reduction of H₂O₂ at the Cₚ of OxyR, unfolding of the Rdx3 helix is required to bring the Cₚ-SOH and Cₚ into close proximity for condensation to a Cₚ-Cₚ disulfide. In the crystal structure of Cg-OxyR₁H₂O₂, two of the four active sites of the tetramer are apo with the Rdx3 unfolded, placing S206 in a position equivalent to the disulfide-engaged C206 (Fig. 4C). This shows that Cg-OxyR can adopt a locally unfolded state without the prerequisite of Cₚ sulfenylation, and a similar observation has been made in the structure of C199D Pa-OxyR (23). In Prdxs, a local unfolding of the Cₚ environment is a well-known phenomenon, and it is associated with an inherent equilibrium between the fully-folded (FF) and locally-unfolded (LU) conformations of the active site (33). Our data suggest that the OxyR Cₚ environment is also subject to FF/LU conformational dynamics, and it is very likely that a FF/LU equilibrium regulates the peroxidoic power of OxyR and susceptibility to Cₚ hyperoxidation. Active-site conformational dynamics have also been observed in a comparative study of Vv-OxyR1 and Vv-OxyR2 (25). Whereas in the majority of OxyRs, the active site His (H205 in C. glutamicum and V. vulnificus) is preceded by a Gly (G204), in Vv-OxyR2 this Gly is substituted with a Glu (G204E), which is proposed to reduce the conformational flexibility of the active site. Due to this active-site rigidity, Vv-OxyR2 reduces H₂O₂ twofold faster than its counterpart Vv-OxyR1 (which contains G204), but it is more susceptible to Cₚ hyperoxidation (25). Despite sharing no conserved structural...
features, it is intriguing to see that both OxyR and Prdx adopted a similar structural dynamic mechanism.

Here we report a significant rearrangement of the tetrameric assembly of Cg-OxyR upon oxidation, yet with localized structural change restricted to the RD subunits only, as the DBD remained unaltered in conformation. The observed shortening of the distance between respective DBD dimer pairs of the oxidized tetramer relative to the reduced form validates the previously hypothesized mechanistic models of oxidative regulation of OxyR in its capacity as a transcription factor (23, 34). See SI Appendix for a hypothesis of how localized structural changes upon Cg-Cr formation in Cg-OxyR drive the transition between the reduced and oxidized tetrameric assemblies. Importantly, these structural changes that occur upon Cg-OxyR oxidation correlate into changes in DNA binding topology and affinity. We show that in the absence of oxidizing stimuli, Cg-OxyR binds to the catalase promoter/operator region at two distinct binding sites upstream and downstream of the katA transcription start point. Upon oxidation, Cg-OxyR loses its interaction with the downstream binding site, and this is further reduced by a reduction in overall affinity and allosteric cooperativity of the binding association between oxidized OxyR and the katA promoter. This is opposite to what has been found for the redox-dependent binding of Ec-OxyR to the katA regulatory region, where the binding affinity increases upon oxidation (34). Although Cg-OxyR oxidation causes loss of binding to the second site, the electrophoretic mobility of the Cg-OxyR-DNA complex is not affected by its redox state (SI Appendix, Fig. S6A), thereby indicating that the same number of Cg-OxyR subunits is associated with the katA regulatory region, regardless of its oxidation state. Furthermore, the decrease in binding cooperativity under reducing conditions suggests that the four DBDs of the Cg-OxyR tetramer bind to the katA regulatory region (two in each binding region), whereas Cg-OxyR oxidation causes a conformational change that leads to the dissociation of two DBDs from the downstream binding region. Because oxidized Cg-OxyR still binds to the −10 promoter element of katA, we can expect a positive interaction between oxidized Cg-OxyR and the RNA polymerase, whereas the extended contacts made with reduced Cg-OxyR might inhibit transcription initiation.

C. glutamicum strains lacking OxyR are more resistant to H2O2 stress, due to catalase derepression (10, 27). Catalase levels are required to survive H2O2 stress, as demonstrated in C. glutamicum and C. diphteriae strains lacking katA, which are hypersensitive to H2O2 (27, 29). We confirm that catalase up-regulation not only improves resistance against elevated H2O2 concentrations, but also shortens the growth lag phase and increases the rate of H2O2 consumption. The strain of C. glutamicum overexpressing Cg-OxyR (∆oxyR + WT Cg-oxyR) exhibits the lowest catalase levels and the lowest survival to sustained H2O2 stress. Under resting conditions, both WT and Cg-OxyR-overexpressing strains show similar repression of katA transcription, but under H2O2 treatment, the strain overexpressing Cg-OxyR exhibits even lower katA mRNA levels, as others have previously observed (27). This likely explains the increased sensitivity of this strain to H2O2 stress. Despite all this, the strain overexpressing Cg-OxyR has one of the highest H2O2 scavenging rates after a single H2O2 bolus. An explanation for this behavior is that OxyR itself is rapidly consuming H2O2, thereby acting as a kind of short-term scavenger. However, OxyR is not a real H2O2 scavenger, as the reductase kinetics of OxyR are too slow to maintain a defense response during prolonged oxidative stress conditions. A H2O2 bolus in E. coli and V. vulnificus causes OxyR oxidation in 30 s, but it takes 5–10 min for OxyR to be re-reduced (19, 35).

In summary, this study provides in vivo evidence for H2O2-dependent transcriptional derepression by Cg-OxyR, and for redox-dependent associations with cognate DNA. With in-depth crystallographic and biochemical analyses, we uncovered the molecular mechanism of hydrogen peroxide sensing by OxyR. OxyR employs a disulfide-driven allosteric structural change at its RD interface. The obtained structural and mechanistic insights might further steer the design of H2O2-sensing inhibitors and guide the optimization of a new generation of HyPer-like redox biosensors.

Methods

Crystallography and X-Ray Diffraction. All crystals of Cg-OxyR were obtained using the hanging-drop vapor diffusion method at 283 K. Cg-OxyR(C206S) was crystallized by mixing 1.5 μL of protein [3 mg mL−1 in 20 mM Tris HCl, pH 8.0, 500 mM NaCl, and 2 mM Tris(2-carboxyethyl)phosphine (TCEP)] with 1.5 μL of 25% PEG 4000, 0.1 M 2-(N-morpholino)ethanesulfonic acid (Mes), pH 6.2, 0.125 M lithium sulfate, 3% 1,6-hexanediol, and 2 mM TCEP. Crystals were cryoprotected by addition of 16% ethylene glycol, 10% glycerol, 10% propanediol, and 2 mM TCEP and flash-frozen. Diffraction data were collected at the PROKIMA-2A beamline of SOLEIL synchrotron at 100 K and a wavelength of 0.98 Å.

To obtain a H2O2-bound protein crystal, 1.65 μL of artificial mother liquor containing an increased concentration of PEG (30% PEG 4000, 0.1 M Mes, pH 6.2, 0.125 M lithium sulfate) was added to a 3.3 μL crystallization droplet of OxyR(C206S), prepared as described above, followed by direct addition of 0.5 μL 6.5 M H2O2. Crystals were harvested at 283 K and flash-frozen for 90 min. Crystals were flash-frozen and diffraction data collected at beamline I24 of the Diamond Light Source synchrotron at 100 K and a wavelength of 1.0 Å.

Cg-OxyR(C206S) was obtained by mixing 1.5 μL of protein (2.5 mg mL−1) with 1.5 μL of 13% PEG 4000, 0.1 M sodium cacodylate, pH 6.5, 0.1 M magnesium acetate and 10 mM ß-Nicotinamide adenine dinucleotide hydrate. Crystals were cryoprotected by addition of 20% ethylene glycol, 15% sucrose, and 15% 3-(1-Pyridinio)-1-propanesulfonate and flash-frozen. Diffraction data were collected at the ID308 beamline of the European Synchrotron Radiation Facility at 100 K and a wavelength of 0.954 Å.

All diffraction data reduction was performed in XDS (36) and merging of intensities in AIMLESS (37). Phases for OxyR(C206S) were determined by molecular replacement using Phaser (38) with full-length P. aeruginosa C199D OxyR (PDB ID code 4X6G) as a search model (23), and the full-length model or RD homodimer of OxyR(C206S) used as a search model in molecular replacement for OxyR(C206S) and OxyR respectively. Atomic coordinates were manually corrected in COOT (39), and the final maximum-likelihood refinement performed in PHENIX (40). Molecular geometry was validated using MolProbity (41), and the Ramachandran outliers for OxyR(C206S), OxyR(C206S)2, and OxyR were 0.08%, 0.17%, and 0% respectively. X-ray data collection and refinement statistics are summarized in Table 1. For a discussion of ligand assignment/fitting to residual density, we direct readers to the SI Appendix. All structural figures were prepared in PyMOL. Oligomeric assemblies of crystal structures were verified using PISA (42).

Methods describing stopped-flow analysis, FOX assay, DNA-binding experiments, generation of mutant strains, transcriptional analysis, and in vivo resistance to H2O2 are described in detail in SI Appendix, Supplemental Materials and Methods.

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