Crystal Structures of the Reduced, Sulfenic Acid, and Mixed Disulfide Forms of SarZ, a Redox Active Global Regulator in Staphylococcus aureus*□

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SarZ is a global transcriptional regulator that uses a single cysteine residue, Cys13, to sense peroxide stress and control metabolic switching and virulence in Staphylococcus aureus. SarZ belongs to the single-cysteine class of OhrR-MgrA proteins that play key roles in oxidative resistance and virulence regulation in various bacteria. We present the crystal structures of the reduced form, sulfenic acid form, and mixed disulfide form of SarZ. Both the sulfenic acid and mixed disulfide forms are structurally characterized for the first time for this class of proteins. The Cys13 sulfenic acid modification is stabilized through two hydrogen bonds with surrounding residues, and the overall DNA-binding conformation is retained. A further reaction of the Cys13 sulfenic acid with an external thiol leads to formation of a mixed disulfide bond, which results in an allosteric change in the DNA-binding domains, disrupting DNA binding. Thus, the crystal structures of SarZ in three different states provide molecular level pictures delineating the mechanism by which this class of redox active regulators undergoes activation. These structures help to understand redox-mediated virulence regulation in S. aureus and activation of the MarR family proteins in general.

Gram-positive Staphylococcus aureus is responsible for a multitude of human illnesses, including abscesses, toxic shock syndrome, and endocarditis, and is a major source of hospital-acquired infections (1, 2). The SarA protein family is one of the major groups of global regulators that coordinate the expression of virulence factors in S. aureus (3). Within the SarA family, a SarA homologue, SarZ, has been identified as a global regulator and shown to affect the expression of nearly 90 genes (4, 5).

S. aureus SarZ is a transcriptional regulator that has affinity for various promoter fragments, including those of ohr, hla, and agr, genes involved in organic hydroperoxide resistance, hemolysin production, and virulence regulation, respectively (4, 6). Transcription profiling identified sarZ as a regulator of metabolic processes, antibiotic resistance, autolysis, peroxide response, and virulence (4). In studies using silkworm and mouse infection models, a sarZ mutant strain produced less hemolysin and exhibited less virulence than the wild-type strain (6). Most recently, SarZ has been identified as a regulator important for the spread of active infections, influencing toxin and protease production (7, 8). Further emphasizing the effect sarZ has on bacterial virulence, a SarZ homologue has been identified in Staphylococcus epidermidis and shown to influence biofilm formation, hemolysis, and protease secretion (9).

SarZ belongs to a superfamily of the MarR-type transcriptional regulators. Proteins in the MarR (multiple antibiotic resistance regulator) family sense and respond to changing environments, controlling genes regulating resistance to antibiotics, oxidative stress agents, detergents, and organic solvents (10). Recently, we have shown that SarZ is a redox active regulator (4). SarZ binding to DNA is disrupted by oxidation with either hydrogen peroxide or organic hydroperoxides. A single cysteine residue (Cys13) in SarZ is sensitive to oxidation and forms a sulfenic acid (Cys-SOH) when exposed to cumene hydroperoxide (CHP)3 in vitro.

Single cysteine oxidation as a mechanism of induction is shared by other members of the MarR family, including S. aureus MgrA and Bacillus subtilis OhrR (BsOhrR) (11–13). Like SarZ, MgrA is a global regulator that influences genes involved in antibiotic resistance and virulence, among others (11, 14–17). An mgrA mutant strain of S. aureus exhibits a dramatic reduction of virulence in a mouse abscess model of infection (11). BsOhrR regulates resistance to organic hydroperoxides by binding to ohrA promoter sequences (12). The lone cysteine in each of SarZ, MgrA, and BsOhrR forms sulfenic acid when exposed to oxidants. However, sulfenic acid form-

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§ The atomic coordinates and structure factors (codes 3HSE, 3HRM, and 3HSR) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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3 The abbreviations used are: CHP, cumene hydroperoxide; BsOhrR, B. subtilis OhrR; XcOhrR, X. campestris OhrR; ESI-FITMS, electrospray ionization-fourier transform mass spectrometry; BT, benzene thiol; LMW, low molecular weight; NBD-CI, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole; DTT, dithiothreitol; HPLC, high pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid; bis-Tris, 2-[bis(2-hydroxyethyl)-amino]-2-(hydroxymethyl)propane-1,3-diol.
tion has not been shown to be sufficient for derepression. In fact, a recent study on BsOhrR suggests that further cysteine modification, to form a mixed disulfide, generate a cyclic sulfenamide, or produce sulfenic (SO2H) or sulfonic (SO3H) acid, is necessary for release from DNA; in particular, formation of a mixed disulfide with small molecule thiols present in the bacterial cytoplasm might be the dominant biological modification (18–20). By contrast, other bacterial peroxide sensors are activated by formation of intra- or intermonomer disulfide bonds including *Escherichia coli* OxyR, *Pseudomonas aeruginosa* MexR, and *Xanthomonas campestris* OhrR (XcOhrR) (21–24).

Crystal structures of both MgrA and BsOhrR have provided insight into the apo and DNA-bound states of the single cysteine class of OhrR-MgrA proteins (11, 25). Both MgrA and BsOhrR were crystallized in their reduced forms (with the cysteine of BsOhrR mutated to a serine to mimic the reduced state), which reveal the reactive cysteine (Cys12 in MgrA and Cys15 in BsOhrR) located in a hydrophobic pocket near the N terminus of helix α1. The DNA-bound BsOhrR is the first structure of a MarR family protein bound to DNA and shows BsOhrR binding to DNA via a winged helix-turn-helix motif in each monomer of the homodimer. To have a full understanding of the oxidation activation mechanism, a structure from the single cysteine class of OhrR-MgrA proteins in an induced state is necessary.

In the present study, we sought to describe the mechanism by which SarZ is activated. Here, we provide crystal structures of SarZ in three different states: reduced, sulfenic acid-modified, and disulfide-modified with an external small molecule thiol. The three structures delineate the mechanism by which SarZ undergoes activation. Very few differences are present between the reduced and sulfenic acid-modified structures, but a large conformational shift in the DNA-binding domains is seen when SarZ is further modified to the mixed disulfide form. *In vitro* analyses of SarZ confirm these cysteine modifications. Overall, this study describes a detailed mechanism by which SarZ is activated through reaction of its lone cysteine. It provides insight into the regulation of OhrR-MgrA proteins and gives a structural framework for future design of small molecule inhibitors to modify the functions of these proteins, in particular MgrA and SarZ from *S. aureus.*

**EXPERIMENTAL PROCEDURES**

**SarZ Expression and Purification**—SarZ protein was produced by cloning sarZ (GenBank™ accession number BAFl6558) into pET28a (Novagen) for overexpression in *E. coli* BL21 Star (DE3) (Invitrogen). A truncated version of SarZ was constructed to improve the diffraction quality of crystals; SarZ (7–142) lacks both the first and last six residues of the protein but contains an N-terminal sequence of Gly-Ser-His-Met introduced by the thrombin and restriction enzyme cutting sites. The cells were grown at 37 °C, with shaking at 250 rpm, in LB medium supplemented with 50 μg/ml kanamycin to an A600 of 0.6–0.9, at which time protein expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside for 4 h at 30 °C. Cells were harvested by centrifugation and stored at −80 °C until use. A cell pellet was suspended in 20 mM Tris (pH 7.5), 300 mM NaCl, 2 mM DTT, and 10% glycerol and lyzed at 4 °C by sonication. After centrifugation to remove cell debris, the lysate was loaded onto a HisTrap HP (GE Healthcare) column, and the protein was eluted with a linear gradient of 0–400 mM imidazole. The His tag was removed by thrombin cleavage, and the protein was passed through a HisTrap HP column again to separate any undigested protein. SarZ was further purified by cation exchange chromatography using a Mono S column (GE Healthcare) and gel filtration chromatography using a Superdex 200 column (GE Healthcare). Selenomethionine-substituted protein was produced by inhibiting methionine biosynthesis and purified as above (26). The cysteine to serine mutation was introduced using the QuickChange mutagenesis kit (Stratagene), and SarZ C13S was purified as above.

**NBD Assay**—Purified SarZ was reduced with 10 mM DTT for 1 h. To generate the sulfenic acid, benzene thiol, or cysteine modifications, SarZ was exchanged into buffer containing a 4-fold molar excess of cumene hydroperoxide, 1 mM benzene thiol, or 1 mM free cysteine, respectively. Cumene hydroperoxide was added to the benzene thiol and cysteine samples. All of the samples were washed three times, adjusted to 50 mM, and incubated with 1 mM 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) in the dark for 1 h. After extensive washing, spectra were taken using an Agilent 8453 UV-visible spectrophotometer.

**ESI-FTMS**—Modified SarZ samples were prepared as above, and molecular weight was determined using liquid chromatography ESI-FTMS. The samples were subjected to reverse phase HPLC through a C8 column (2.1 × 75 mm Poroshell 300SB-C8; Agilent) with a gradient of 18.5–54.5% acetonitrile in 0.1% formic acid over 60 min. HPLC peaks were analyzed using a hybrid linear ion trap fourier transform-ion cyclotron resonance mass spectrometer (LTQ-FT; Thermo Scientific). The average masses were computed with the XTractAll for Qual Browser program (Thermo Electron).

**Crystallization and Structure Determination of SarZ-SH and SarZ-SOH**—Purified SeMet-SarZ in buffer A (10 mM MES, pH 6.6, 200 mM NaCl) crystallized at room temperature using the hanging drop vapor diffusion method and a reservoir solution of 0.1 M bis-Tris (pH 5.5) and 25% polyethylene glycol 3,350. SarZ-SOH crystals appeared within 1 day and were frozen in N2 (l) following cryoprotection with the reservoir solution containing 20% glycerol. The data were collected to 2.3 Å at the General Medicine and Cancer Institutes Collaborative Access Team Beamline 23-ID-B at the Advanced Photon Source, Argonne National Laboratory, and processed using HKL2000 (27). The structure was determined by single-wavelength anomalous dispersion from four selenium atoms in the asymmetric unit cell. Initial phases were calculated with SOLVE (28) and then improved with RESOLVE (29). The model was built using the program Coot (30) and refined with CNS (31).

Reduced SarZ in buffer A (with 10 mM DTT) crystallized in 0.1 M citric acid (pH 3.0) and 3.2 M ammonium sulfate. The crystals were cryoprotected with the reservoir solution containing 20% glycerol and frozen in N2 (l). The data were collected to 2.9 Å at the Structural Biology Center Beamline 19BM at the Advanced Photon Source and processed using HKL2000. The phases were determined using Phaser (32) from the CCP4 software suite (33) and searching for two copies of monomer A.
from SarZ-SOH. The model was built and improved as above, using Coot and CNS.

**Cystallization and Structure Determination of SarZ-BT**—Purified SarZ in buffer A (with 2 mM DTT) was concentrated to 350 μM in an Ultrafree-0.5 centrifugal filter device (Millipore), and the buffer was exchanged twice using a Micro Bio-Spin Chromatography column (Bio-Rad) with 100 mM potassium phosphate (pH 7.0), 200 mM NaCl, 1 mM EDTA, and 1 mM benzene thiol. SarZ was incubated with a 10-fold molar excess of cumene hydroperoxide at room temperature for 15 min. The solution was washed with buffer A (without DTT) three times, and SarZ was concentrated to 10 mg/ml for crystallization experiments.

SarZ-BT was crystallized at room temperature using the hanging drop vapor diffusion method and a reservoir solution of 0.1 M Tris (pH 8.5), 0.2 M ammonium acetate, and 25% polyethylene glycol 3,350. Rod-shaped crystals appeared within 2 days and were frozen in N2 (l) following cryoprotection with the reservoir solution containing 16% glycerol. The data were collected to 1.9 Å at the General Medicine and Cancer Institutes Collaborative Access Team Beamline 23-ID-B at the Advanced Photon Source and processed using HKL2000 (27). Phases were determined using Phaser (32) from the CCP4 software suite (33) and searching for one copy of the dimerization domain from SarZ-SOH (residues 3–30 and 106–141 from monomer A and residues 3–30 and 106–139 from monomer B) and two copies of the DNA-binding domain (residues 33–104 from monomer A). The structure was built using the program Coot (30) and refined with Refmac5 (34) from the CCP4 suite. The figures were prepared with PyMOL (35). All of the crystallographic data and refinement parameters are summarized in supplemental Table S1.

**RESULTS**

**Cys\(^{13}\) Modification in SarZ**—We have shown that SarZ is a transcriptional regulator affecting the expression of many genes involved in the virulence of *S. aureus* by sensing oxidative stress through its lone cysteine residue, Cys\(^{13}\) (4). Although Cys\(^{13}\) can be oxidized to sulfenic acid, the oxidation state necessary for release from DNA has been shown to be S-thiolation. Here, we tested whether Cys\(^{13}\) could be stably S-thiolated by either cysteine or benzene thiol (BT) under oxidative stress conditions.

SarZ was oxidized by cumene hydroperoxide (CHP), reacted with a low molecular weight (LMW) thiol molecule, and analyzed for modification by both the NBD assay and mass spectrometry. In the NBD assay, the presence of reduced thiols and sulfenic acids can be evaluated by reaction with NBD-CI because the reduced and oxidized adducts absorb at different wavelengths (36). Reduced SarZ produces a peak at 420 nm, and CHP-oxidized SarZ produces a peak at 347 nm, consistent with SarZ-S-NBD and SarZ-SO-NBD formation, respectively (Fig. 1A). However, when SarZ is oxidized in the presence of free cysteine or benzene thiol, no peak is observed, indicating the lack of reduced thiol or sulfenic acid moieties and suggesting the formation of a disulfide bond between Cys\(^{13}\) and the external thiol.

We performed ESI-FTMS, which confirmed formation of disulfide-linked SarZ with either cysteine or BT (Fig. 1, B–D, and Table 1). When SarZ was treated with CHP in the presence of an excess of free cysteine or BT, a mixed disulfide bond formed. A mass increase of 119 Da was observed in the cysteine-treated sample, indicating a 1:1 stoichiometry of SarZ monomer and external cysteine. Upon reaction with BT, −74% of SarZ was modified by BT, as shown by a mass increase of 108 Da.

**Structure of Reduced and Sulfenic Acid Forms of SarZ**—The reduced (SarZ-SH) and sulfenic acid forms (SarZ-SOH) of SarZ were crystallized in the presence and absence of a reducing agent, respectively. The structure of SarZ-SOH was determined using single-wavelength anomalous dispersion data from selenomethionine-substituted protein crystals and that of SarZ-SH was determined by molecular replacement using a monomer of SarZ-SOH as the search model. Reduced SarZ was crystallized in space group P4\(_2\)2\(_1\) and refined to 2.9 Å; SarZ-SOH was crystallized in space group P2\(_1\)2\(_1\)2\(_1\) and refined to 2.3 Å. SarZ-SH and SarZ-SOH each had two monomers in the asymmetric unit, forming the biologically relevant dimer.

The overall structure of SarZ comprises a homodimer adopting the triangular shape found in MarR family proteins (Fig. 2). The structure is helix-rich, with the following arrangement of secondary structural elements: α1-α2-β1-α3-α4-β2-β3-α5-α6. The two monomers associate via a dimerization interface provided by helices α1, α5, and α6, and each monomer contains a winged helix-turn-helix DNA-binding domain provided by β1, α3, α4, β2, and β3. The three β strands form an antiparallel β-sheet. Helix α4 is the recognition helix in the helix-turn-helix motif, and the wing is defined by strands β2 and β3 and their connecting loop. Residues of the connecting loop of the wing could not be modeled in the structures, indicating the flexibility of this region. In the structure of BsOhrR bound to DNA (25), the wing makes many contacts with DNA; therefore, the disorder of the wings of SarZ is not unexpected in the absence of bound DNA.

As in MgrA and BsOhrR, the reactive cysteine of SarZ is located toward the N terminus of helix α1. In the oxidized SarZ structure, the sulfenic acid modification of Cys\(^{13}\) can be clearly viewed in the electron density (Fig. 3). Originally, this residue was modeled as a reduced cysteine; as refinement progressed, a blob of positive electron density appeared within covalent bond distance of Sy. The inclusion of an oxygen atom in the model, built with 100% occupancy and refined to a B-factor consistent with other atoms in the residue and area, satisfied the positive electron density. Cys\(^{13}\) is in a pocket formed by residues from the other monomer on helices α1’, α2’, and α5’. An extensive hydrogen bond network surrounds Cys\(^{13}\) and involves residues Ser\(^{113}\), Tyr\(^{277}\), Tyr\(^{386}\), Tyr\(^{411}\), and Cys\(^{13}\) (Fig. 4A). In reduced SarZ, Cys\(^{13}\) forms a hydrogen bond with Tyr\(^{277}\) and a water-mediated hydrogen bond with Tyr\(^{386}\). When Cys\(^{13}\) is oxidized to a sulfenic acid, the same hydrogen bonds are formed with Oδ instead of Sy (Fig. 4B and supplemental Fig. S1).

The reaction of Cys\(^{13}\) to form a mixed disulfide is proposed to proceed from the reduced thiol to a sulfenic acid, which acts as an electrophile to react further with free LMW thiols (37). The first step, in which cysteine is oxidized by a peroxide, depends on the ionization state of the residue. The thiolate anion is more nucleophilic than a protonated thiol. Therefore, a reactive cys-
tein should have a lowered sulfhydryl pK_a. Indeed, an analysis of proteins with known sulfenic acid modifications found a pK_a decrease of greater than 1 unit compared with a control set (38), and the pK_a of Cys15 in BsOhrR was found to be 5.2, ensuring its deprotonation at physiological pH (25). A similar lowering of the pK_a of the Cys13 of SarZ is expected and may be accounted for by two factors. First, there are numerous basic residues in the vicinity (supplemental Fig. S2). Lysine 10 is one helical turn and 10 Å away from Cys13, and lysines 21 and 25 are two and three helical turns distant, respectively. Although the distance between Cys13 and any of the lysine residues is fairly large, precluding a major impact on Cys13 reactivity, the presence of the basic side chains could contribute. Second, and perhaps most important, the hydrogen bond network surrounding Cys13 can stabilize the thiolate (38). A recent mutation study of BsOhrR, which contains a single reactive cysteine that is sensitive to oxidation, has shown that the tyrosine residues forming hydrogen bonds with the reactive Cys residue are essential for its reactivity (39). Once formed, the sulfenic acid modification could be stabilized by both hydrogen bonds and the lack of local reduced cysteines (40). The sulfenic acid side chain in oxidized SarZ forms two hydrogen bonds through its oxygen atom, which may contribute to its stability. As we have demonstrated, Cys-SOH reacts with available LMW thiols to form mixed disulfides. The absence of such thiols under our crystallization conditions, either free in solution or located elsewhere in the protein, allows the characterization of the sulfenic acid in the oxidized SarZ.

Overall, the structures of both reduced and sulfenic acid forms of SarZ are very similar; superimposition of the two dimers results in a root mean square deviation of only 1.16 Å (Fig. 5A). The spacing between the DNA-binding domains, measured between the middle of recognition helices α4 and α4’, is 32 and 30 Å for reduced and sulfenic acid-modified SarZ, respectively. Although not the canonical 34 Å apart, this spacing is suggestive of a configuration compatible with the recognition helices binding to consecutive major grooves of B-DNA. In fact, in the BsOhrR-DNA structure, BsOhrR bends and undertwists DNA, shortening the distance between consecu-

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**TABLE 1**

| Sample | Component (% of relative abundance) | Observed molecular mass (Da) | Structure |
|--------|----------------------------------|-----------------------------|-----------|
| SarZ   | A (100)                          | 16386.70                    | SarZ (Cys13–SH) |
| SarZ C13S | A (100)                         | 16370.71                    | SarZ (Ser13–OH) |
| SarZ + BT + CHP | A (26)                      | 16386.70                    | SarZ (Cys13–S-BT) |
| SarZ + Cys + CHP | B (74)                  | 16494.70                    | SarZ (Cys13–S–S–Cys) |

*Calculated based on height of peak in total ion count.*
tive major grooves and allowing its recognition helices, separated by 31 Å, to bind (25). We propose a similar mechanism, in which SarZ binds DNA by slightly distorting it (Fig. 5B). Most importantly, the structural evidence indicates that SarZ can bind DNA with Cys13 either reduced or oxidized to sulfenic acid. This structural feature of the sulfenic acid form of SarZ confirms previous observations for this family of proteins (19).

Structure of BT-modified SarZ—Crystals of BT-modified SarZ (SarZ-BT) were grown after oxidizing SarZ in the presence of benzene thiol. The structure of SarZ-BT was solved by molecular replacement using domains from SarZ-SOH as the search model. SarZ-BT was crystallized in space group P1 and refined to 1.9 Å. Four monomers, forming two dimers, were found in the asymmetric unit. There are three molecules of BT in the structure, forming disulfide bonds with Cys13 in three of the four monomers, which is consistent with the mass spectrometry data indicating 74% modification of SarZ sample upon BT reaction. Dimer AB contains one molecule of BT, and dimer CD contains two; therefore, we will focus our analysis on dimer CD, hereafter referred to as SarZ-BT.

The structure of SarZ-BT differs from SarZ-SH and SarZ-SOH, ultimately leading to a conformation that is less compat-

FIGURE 2. Structures of reduced, sulfenic acid, and disulfide-modified SarZ, and sequence alignment. A, reduced SarZ structure with one monomer colored blue and the other colored gray. Cys13 is shown as yellow sticks. B, sulfenic acid form of SarZ structure with one monomer colored red and the other colored gray. Cys13-SOH is shown as yellow sticks. C, benzene thiol-modified SarZ structure with one monomer colored red and the other colored gray. Cys13 and BT residues are shown as yellow sticks.

FIGURE 3. Electron density of the SarZ sulfenic acid modification. In SarZ-SOH, Cys13 is modeled as a thiol (A) or sulfenic acid (B). The positive electron density (blue) seen in A is satisfied by the sulfenic acid modification of B. Electron density maps of A are simulated annealing omit maps, with the oxygen of the sulfenic acid omitted. Fo − Fc maps, at 1.5 σ, are colored tan, and the Fo − Fc map, at 2.5 σ, is colored blue. The atoms are colored light blue (carbon), dark blue (nitrogen), red (oxygen), and yellow (sulfur).

FIGURE 4. Reactive Cys13 pocket. A, reduced Cys (SH). B, sulfenic acid Cys (SOH). Atoms are colored gray (carbon), red (oxygen), and yellow (sulfur). Water molecules are labeled W1 or W2 and shown as red spheres. Hydrogen bonds are shown as black dashed lines. A, Ser113 to Tyr41 3.8 Å; Ser113 to Tyr27 2.9 Å; Cys13 to Tyr27 3.3 Å; Cys13 to W2 3.0 Å; W1 to Tyr38 2.6 Å. B, Ser113 to Tyr41 3.5 Å; Ser113 to Tyr27 2.7 Å; Cys13-SOH O6 to Tyr27 3.0 Å; Cys13-SOH O6 to W1 2.7 Å; W1 to Tyr38 2.6 Å; W2 to Cys13-SOH S 3.7 Å.
S. aureus Regulator SarZ Structures

Both the hydrogen bond network and the organization of residues in the cysteine pocket are disrupted by disulfide bond formation. In SarZ-SH and SarZ-SOH, Sy of reduced Cys and O6 of sulfenic acid each makes two hydrogen bonds. The analogous hydrogen bonds are mostly absent in SarZ-BT. In monomer C, Sy of Cys forms only one weak contact with Tyr, (Fig. 6A). This hydrogen bond is not expected to be as strong as those observed in SarZ-SH or SarZ-SOH because it is between a tyrosine and a persulfide as opposed to between a tyrosine and either a thiol (or thiolate) or a sulfenic acid (or sulfenate). No hydrogen bonds are observed between Sy of Cys and the sulfur atom of benzene thiol and the surrounding residues in monomer D (Fig. 6B). The addition of BT to the cysteine pocket also causes a reorganization of Phe, located at the C terminus of helix α5 in the other monomer. In SarZ-SH and SarZ-SOH, Phe is directed toward Cys, buried in the dimerization interface. With BT oriented as in monomer D, Phe and BT would sterically clash (Fig. 6C). Phe in SarZ-BT is therefore flipped into the opposite orientation, allowing BT to replace it in the hydrophobic pocket.

The sulfenic acid form of the BsOhrR-MgrA type proteins (one cysteine) is proposed to further react with a LMW thiol present in the bacterial cytoplasm to form a mixed disulfide bond, which triggers a structural change that leads to dissociation of the protein from DNA. The relevant available thiol in B. subtilis and S. aureus include coenzyme A and cysteine, because neither organism produces glutathione. Although benzene thiol is not a biological LMW thiol, its modification of Cys in SarZ does give a disulfide-modified protein that provides important structural insights into this activation mechanism. The BT-modified SarZ structure confirms the

**Figure 6. Cys′ pocket in SarZ-BT.** A and B, BT-modified Cys′ of monomer C (A) and D (B). Atoms are colored gray (carbon), red (oxygen), and yellow (sulfur). Hydrogen bonds are shown as black dashed lines. A, Ser113 to Tyr, 3.8 Å; Ser113 to Tyr, 2.6 Å; Cys13-BT Sy to Tyr, 3.3 Å; B, Ser113 to Tyr, 2.9 Å; Ser113 to Tyr, 2.5 Å. C, overlay of SarZ-SH (blue), SarZ-SOH (green), and SarZ-BT (red) showing the steric clash between Cys-BT and Phe117 from either SarZ-SH or SarZ-SOH. In the SarZ-BT structure, Phe117 is flipped away from the modification.

The organization of the cysteine pocket is different in the two monomers of SarZ-BT (Fig. 6). In monomer C, BT is orientated toward the surface of the protein, projecting into a cavity between the dimerization and DNA-binding domains. Conversely, BT of monomer D is buried in a hydrophobic pocket in the dimerization domain (Fig. 6B). The benzene ring of BT in monomer D is surrounded by Leu, Phe, Ile, Phe, Ile, and Phe. The orientation of the two disulfide bonds may provide a fortuitous look at the path of the reaction mechanism. Benzene thiol needs to approach Cys′ SOH from the direction in which it is oriented in monomer C (supplemental Fig. S3A); this is the side of the cysteine pocket that is accessible to solvent. After reaction, a change in the rotamer conformation of Cys′ and rotation around the disulfide bond yield the orientation of monomer D (supplemental Fig. S3B), which is probably more thermodynamically stable given the hydrophobicity of BT and the surrounding residues.

**Figure 5. Structural implication of SarZ modification for binding to DNA.** A, superimposition of the dimers of SarZ-SH (blue) and SarZ-SOH (green) highlights their similarity. B, superimposition of SarZ-SOH (green) and the BsOhrR-DNA (orange-purple) complex shows that SarZ-SOH is preconfigured for binding to DNA. C, superimposition of a single monomer of SarZ-SOH (green) and SarZ-BT (red). The other monomers of the dimers are aligned to highlight the changes in the monomers shown. D, superimposition of SarZ-SOH (green) and SarZ-BT (red) on DNA (purple) shows that SarZ-BT adopts a configuration unfavorable to bind DNA. Alignment is along the monomers on the left to highlight the differences in the apposing monomers. Points of steric clash between SarZ-BT and DNA are circled. DNA is modeled based on the BsOhrR-DNA complex structure (25).
observed DNA binding data for the disulfide-modified SarZ (4) and provides molecular level details to explain the attenuated DNA affinity of the modified protein. A combination of losing hydrogen bond interactions surrounding the reactive Cys residue and additional added steric clashes from the mixed disulfide contributes to an allosteric conformational change of the DNA-binding domains of the protein.

**DISCUSSION**

We report here the structural characterization of the global transcriptional regulator SarZ in *S. aureus*. The crystallographic analyses of the reduced, sulfenic acid, and mixed disulfide form of SarZ provide the first complete molecular level pictures of the single cysteine class of redox active OhrR-MgrA regulatory proteins in different states. The sulfenic acid form of this class of proteins is structurally characterized for the first time. This structure confirms that the sulfenic acid form still binds DNA and is an intermediate state on the way to a further modified state in the OhrR-MgrA class of proteins.

Interestingly, the sulfenic acid modification is stabilized by two hydrogen bonding interactions that also exist in the reduced structure (Fig. 4 and supplemental Fig. S1). This stabilization may help prevent irreversible transformation of the sulfenic acid to further oxidized forms, such as sulfinic or sulfonic acid. This allows the sulfenic acid form of the protein to be dominantly modified to a mixed disulfide under peroxide stress, which can then be reversibly converted back to the DNA-binding reduced form when the stress is gone. This cycle could be mechanistically important because SarZ plays a key role in metabolic switching as well as virulence regulation in *S. aureus* (4). To survive inside a complex host, the pathogens may gain advantage by quickly adjusting to the changing environment instead of waiting for newly synthesized regulators.

The crystal structures of many MarR family proteins have been described, including those of MarR, MexR, MgrA, BsoOhrR, and XcOhrR, among others (11, 22, 25, 43–45). Of those, MarR and MexR have been described in complex with an effector molecule (43, 46, 47). MarR from *E. coli* was crystallized with two molecules of salicylate, a known inhibitor of MarR, hydrogen-bonded on either side of its recognition helix (43). With bound salicylate, MarR is unsuitable for binding to DNA because its recognition helices are not in a proper conformation. The structures of both apo and salicylate-bound MTH313, a MarR homologue from *Methanothermobacterthermoautotrophicum*, show an increase in the distance between the DNA-binding lobes when salicylate binds (46). The MexR repressor from *P. aeruginosa* has been crystallized with and without its antirepressor peptide, ArmR (44, 47). Apo-MexR shows considerable variation in the spacing between DNA-binding lobes, ranging from 23 to 29 Å, which led to the supposition that modification of this spacing dictates binding to DNA (44). This supposition was confirmed by the structure of MexR in a complex with ArmR (47). Binding of ArmR leads to a 13 Å shift in the recognition helix, making its binding to DNA improbable. Interestingly, part of the ArmR-binding site of MexR and one of the salicylate-binding sites of MTH313 is in the same place as the reactive cysteine of OhrR, MgrA, and SarZ, indicating a conserved effector site in MarR proteins (46, 47).

With the introduction of the first MarR protein structure, flexibility in the dimerization domain, especially helix α5 and the loop connecting helices α5 and α6, was proposed as accounting for movement of the DNA-binding lobes relative to each other and regulating binding to cognate DNA. Since then, many examples of conformational flexibility have been seen in MarR proteins, and this flexibility is accounted for by the flexibility of connecting loops and the hydrophobic nature of the dimerization domain (45). Indeed, the changes evident in the structures of apo and inducer-bound SarZ are located in the dimerization domain, specifically helix α5 and the loop connecting helices α5 and α6. The movement of Phe117 highlights this. In addition, in the structures of reduced and oxidized OhrR from *X. campestris* (XcOhrR), where its reactive cysteine forms an intermolecular disulfide bond when challenged with oxidant, the second cysteine is at the C terminus of helix α5 in a spot analogous to Phe117 from SarZ (22). This second cysteine undergoes a dramatic rotation and translation to form the disulfide bond, mediated through the dissection of helix α5 into two helices. Although helix α5 in SarZ-BT does not reorganize to the extent of helix α5 in XcOhrR upon modification, it does shorten and bend, suggesting a conserved area of flexibility in the MarR family. Further, a recent study on BsoOhrR converted the one-cysteine protein to a two-cysteine protein by introducing a second cysteine in a spot analogous to that of the second cysteine in XcOhrR (48). When exposed to oxidants, the mutated BsoOhrR formed a disulfide bond. This result suggests that one-cysteine oxidation sensors, like SarZ, MgrA, and BsoOhrR, are able to undergo conformational changes similar to XcOhrR to generate a disulfide bond, and therefore the focus on the flexibility of helix α5 in SarZ is warranted.

The structural data gathered thus far on MarR proteins suggest that there are two mechanisms for derepression, epitomized by XcOhrR and MexR/SarZ. The two mechanisms share a rigid body rotation of the winged helix-turn-helix DNA-binding domains but differ in their orientations of the two domains relative to each other. When reduced XcOhrR is oxidized and forms intersubunit disulfide bonds, the DNA-binding lobes rotate 28°, resulting in a nearly perpendicular orientation of the domains and recognition helices, inducing DNA dissociation (22). On the other hand, when MexR binds its antirepressor peptide, ArmR, its DNA-binding domains undergo a dramatic translation with less of a rotation (47). The translation results in steric clashes with DNA. Similar translations are seen in different apo-MexR dimers and apo- and inducer-bound MTH313 (44, 46). The structure of the disulfide-modified SarZ-BT represents the first characterization of a single-cysteine, OhrR-MgrA class of proteins in an inducer-bound state. We show that it is characterized by the second mechanism of derepression; its DNA-binding domains undergo a significant translation when the protein is modified by an external thiol, which disrupts its ability to bind DNA.
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