Biochemical Characterization of the Structural Zn\(^{2+}\) Site in the *Bacillus subtilis* Peroxide Sensor PerR\(^{\text{Fur}}\)

In *Bacillus subtilis* most peroxide-inducible oxidative stress genes are regulated by a metal-dependent repressor, PerR. PerR is a dimeric, Zn\(^{2+}\)-containing metalloprotein with a regulatory metal-binding site that binds Fe\(^{2+}\) (PerR:Zn,Fe) or Mn\(^{2+}\) (PerR:Zn,Mn). Reaction of PerR:Zn,Fe with low levels of hydrogen peroxide (H\(_2\)O\(_2\)) leads to oxidation of two His residues thereby leading to derepression. When bound to Mn\(^{2+}\), the resulting PerR:Zn,Mn is much less sensitive to oxidative inactivation. Here we demonstrate that the structural Zn\(^{2+}\) is coordinated in a highly stable, intrasubunit Cys\(_4\):Zn\(^{2+}\) site. Oxidation of this Cys\(_4\):Zn\(^{2+}\) site by H\(_2\)O\(_2\) leads to the formation of intrasubunit disulfide bonds. The rate of oxidation is too slow to account for induction of the peroxide stress response by micromolar levels of H\(_2\)O\(_2\) but could contribute to induction under severe oxidative stress conditions. In vivo studies demonstrated that inactivation of PerR:Zn,Mn required 10 mM H\(_2\)O\(_2\), a level at least 1000 times greater than that needed for inactivation of PerR:Zn,Fe. Surprisingly even under these severe oxidation conditions there was little if any detectable oxidation of cysteine residues in vivo: derepression was correlated with oxidation of the regulatory site. Because oxidation at this site required bound Fe\(^{2+}\) in vitro, we suggest that treatment of cells with 10 mM H\(_2\)O\(_2\) released sufficient Fe\(^{2+}\) into the cytosol to effect a transition of PerR from the PerR:Zn,Mn form to the peroxide-sensitive PerR:Zn,Fe form. This model is supported by metal ion affinity measurements demonstrating that PerR bound Fe\(^{2+}\) with higher affinity than Mn\(^{2+}\).

PerR is a member of the Fur family of metal-dependent regulators, and many Fur family proteins contain one Zn\(^{2+}\) ion per monomer in addition to a regulatory metal ion. The x-ray absorption spectroscopy of *Escherichia coli* Fur (Fur\(_{\text{EC}}\)) revealed Zn\(^{2+}\) bound in a tetrahedral environment composed of two S and two N/O donor ligands (4). Subsequent studies using chemical modification and mass spectroscopy assigned Cys\(_{92}\) and Cys\(_{95}\) as Zn\(^{2+}\) ligands (5), a conclusion supported by site-directed mutagenesis studies (6). To date, the only structure available for a protein from the Fur superfamily is *Pseudomonas aeruginosa* Fur (Fur\(_{\text{PA}}\)) that was crystallized in the presence of Zn\(^{2+}\) (7). Fur\(_{\text{PA}}\) contains two metal-binding sites assigned as a high affinity Zn\(^{2+}\)-binding site and low affinity Fe\(^{2+}\)-binding site, respectively, by accompanying spectroscopy. No cysteine residues were involved in metal coordination in Fur\(_{\text{PA}}\), and there is still considerable uncertainty surrounding the functional role of the metal-binding sites. For example, multiple mutants in the corresponding residues in *Bradyrhizobium japonicum* Fur failed to abrogate metal sensing (8). Moreover putative Zn\(^{2+}\) ligands in Fur\(_{\text{PA}}\) correspond to residues in PerR required for binding the regulatory metal ion (Fe\(^{2+}\) or Mn\(^{2+}\)) but not the structurally important Zn\(^{2+}\) (9).

Most PerR-regulated genes are derepressed when cells are exposed to low levels of H\(_2\)O\(_2\) (<10 \(\mu\)M), by limitation for metal ions or by transition into stationary phase during aerobic growth, which leads to an increase in the endogenous production of H\(_2\)O\(_2\) (2, 10, 11). Physiological studies have revealed that PerR-regulated genes are strongly repressed in medium supplemented with manganese (and lacking added iron) and that, under these growth conditions, the ability of H\(_2\)O\(_2\) to induce expression is greatly reduced. In contrast, in iron-containing growth medium PerR repression is rapidly relieved upon exposure to H\(_2\)O\(_2\) (2).

These results led to a model in which PerR could bind either Mn\(^{2+}\) or Fe\(^{2+}\) as corepressor to generate two forms of PerR that differ in their sensitivity to reactive oxygen species. Biochemical analyses of the reconstituted PerR:Zn,Fe and PerR:Zn,Mn forms of the repressor provide an explanation for this difference: the high sensitivity of PerR:Zn,Fe results from the regio-specific oxidation of two His residues directly coordinated to the activating ferrous ion (9). Thus, in contrast with most peroxide sensors characterized to date (12–14), PerR does not appear to use thiol disulfide chemistry to sense oxidative stress despite the presence of four conserved cysteine residues in the protein sequence. Previous results from homology modeling of PerR on the Fur\(_{\text{PA}}\) structure, combined with site-directed mutagenesis, support a model in which these four residues form a high affinity Cys\(_4\):Zn\(^{2+}\) structural site (9).
Although the Cys$_4$Zn$_2^+$ site is not involved in sensing low levels of peroxides, this does not rule out a possible role under conditions of severe oxidative stress. Indeed it has been previously proposed that other PerR homologs sense H$_2$O$_2$ by Cys-based redox reactions as inferred from studies done with 5–10 mM H$_2$O$_2$ (15, 16). Analysis of E. coli HSP33 indicates that cells may contain systems that respond specifically to severe oxidative stress conditions. HSP33 is a redox-regulated protein chaperone that contains a Cys$_2$Zn$_2^+$ site that is only oxidized in the presence of high levels of peroxides (e.g. 4 mM H$_2$O$_2$) and elevated temperatures (e.g. 43 °C) (17).

In the present study, we investigated oxidation of the Cys$_2$Zn$_2^+$ site and its possible role in peroxide sensing in vivo. By monitoring Zn$_2^+$ release, we found a rate constant for the in vitro oxidation of this Cys$_2$Zn$_2^+$ site by H$_2$O$_2$ of ~0.05 mM$^{-1}$s$^{-1}$. Oxidation led to the formation of intramolecular disulfide bonds between cysteine residues in the two CXXC motifs, and the resulting oxidized species could be resolved by SDS-PAGE. The in vitro sensitivity of the Cys$_2$Zn$_2^+$ site to oxidation suggests that this site could mediate inactivation of the PerR:Zn,Mn form of the repressor under severe oxidative stress conditions. However, even when treated with high levels of H$_2$O$_2$ there was little if any detectable cysteine oxidation in vivo, and derepression was correlated with oxidation of the regulatory site. Because PerR bound Fe$_2^+$ with higher affinity than Mn$_2^+$, we propose a model in which high levels of H$_2$O$_2$ release sufficient Fe$_2^+$ into the cytosol to effect a transition of PerR from the PerR:Zn,Mn form to the peroxide-sensitive PerR:Zn,Fe form.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—All of the chemicals used were reagent grade. The disodium salt of 4-acetamido-4′-maleimidystilbene-2,2′-disulfonic acid (AMS)$^2$ was purchased from Molecular Probes (Eugene, OR). Anti-FLAG M2-alkaline phosphatase, anti-FLAG M2 affinity gel, anti-rabbit IgG-probe (Eugene, OR). Anti-FLAG M2-alkaline phosphatase, anti-FLAG M2 affinity gel, anti-rabbit IgG-probe (Eugene, OR).

**Determination of Protein Concentration**—The concentration of proteins in the cell crude extract was determined by Bio-Rad protein assay and/or Bio-Rad DC protein assay (for glucose, 2 g/liter (NH$_4$)$_2$SO$_4$, 0.2 g/liter MgSO$_4•7$H$_2$O, 1 g/liter sodium citrate-2H$_2$O, 1 g/liter potassium glutamate, 10 mg/liter tryptophan, 3 mM (NH$_4$)$_6$Mo$_7$O$_24$, 400 mM H$_2$BO$_3$, 30 mM CoCl$_2$, 10 mM CuSO$_4$, 10 mM ZnSO$_4$, and 80 mM MnCl$_2$.

**Construction of PerR-overexpressing E. coli Strains**—The perR open reading frame was PCR-amplified using forward primer 5′-GGTGCATGACCCCATGGCCTGACAT-3′ and reverse primer 5′-TGACCTTTGTCGGATCCGGCTTAA-3′, creating Ncol and BamHI sites, respectively (underlined), with B. subtilis CU1065 chromosomal DNA as template. For the construction of the PerR-FLAG-overexpressing E. coli strain, forward primer 5′-AAGAGAGTGCATGACCCCATGGCCTGACAT-3′ and reverse primer 5′-GATGACCTCGGATCCGGGAATTGAGC-3′, creating Ncol and BamHI sites, respectively (underlined), were used with HB9612 genomic DNA (see below) as template. These PCR fragments were cloned into the Ncol and BamHI sites of expression vector pET16b (Novagen) resulting in plasmids named pJL041 and pJL069, respectively. These plasmids were introduced into E. coli BL21 (DE3) pLysS resulting in PerR- and PerR-FLAG-overexpressing E. coli strains named HE9501 and HE9526, respectively.

**Purification of PerR after Overexpression in E. coli**—E. coli cells were harvested from the 10 ml of overnight culture in LB medium and inoculated into 1 liter of fresh LB medium containing 0.4% glucose, 34 mg/liter chloramphenicol, and 100 mg/liter ampicillin. Isopropyl 1-thio-β-D-galactopyranoside was added to final concentrations of 1 mM at A$_{600}$ of ~0.6, and the cells were allowed to grow for an additional 2 h. Cells were harvested by centrifugation, and the cell pellets were resuspended in 50 ml of buffer A (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 5% (v/v) glycerol) containing 10 mM EDTA. After lysis by sonication, cell debris were removed by centrifugation, and the resulting supernatant was loaded onto a 20-ml heparin-Sepharose column. After application of a linear gradient of 0.1–1 M NaCl, PerR-containing fractions were pooled and buffer-exchanged to buffer A containing 10 mM EDTA using a YM-10 ultrafiltration membrane under the pressure of N$_2$ gas. The resultant PerR was applied to a Mono Q column using an FPLC system (GE Healthcare) and separated using a linear gradient of 0.1–1 M NaCl. The PerR-containing fractions were further purified on a Superdex 200 HiLoad 16/60 column (GE Healthcare) equilibrated with Chelex-100-treated buffer A without EDTA. Purification of PerR was also carried out as above in the absence of EDTA or in the presence of DTT. Active PerR (purified in the presence of 10 mM EDTA) contained ~1.6 mol of zinc/dimer as judged by inductively coupled plasma MS and could be reconstituted with Mn$_2^+$ to give protein with ~60% active molecules in a DNA binding assay. PerR purified in the absence of EDTA was also dimeric and contained stoichiometric zinc (~1.9 mol of zinc and ~0.1 mol of iron/dimer) but was largely inactive due to oxidation of the regulatory metal site as reported previously (9).

**Determination of Protein Concentration**—The concentration of purified PerR was determined by measuring A$_{277}$ nm using the calculated value of e$_{277}$ nm = 10,400 M$^{-1}$ cm$^{-1}$. The concentration of proteins in the cell crude extract was determined by Bio-Rad protein assay and/or Bio-Rad DC protein assay (for
samples containing SDS) using bovine serum albumin as standard.

Construction of FLAG-tagged PerR in B. subtilis—A partial perR gene corresponding to about 350 bp from the 3‘-end of perR excluding the stop codon was amplified by PCR using forward primer 5’-GCAGTACGCTTGGGAAAGAAATTCTCTACTGAGC-3’ and reverse primer 5’-GCACGGCCGATTTTCTTTTTCGAAACCTTGGCGACGAC-3’ with B. subtilis CU1065 chromosomal DNA as template. These PCR fragments were cloned into the KpnI and EagII sites of epitope protein-tagging integration vector pMUTIN-FLAG (18) resulting in plasmid pJL062. This plasmid was passed through E. coli JM105 (recA), and the resulting plasmids were used for transformation of B. subtilis CU1065. The generation of perR-FLAG, upon integration of pJL062 into perR, was verified by PCR followed by sequencing, and the resulting strain was named HB9612. The perR-FLAG open reading frame was PCR-amplified using forward primer 5’-CCATGTAGCGAAAAGCTTCAAACCC-3’ and reverse primer 5’-GTTTCCACCGGAATTCCTTGCGATTTG-3’ with HB9612 genomic DNA as template. These PCR fragments were cloned into the HindIII and EcoRI sites of pDG1730 resulting in plasmid pJL062. The ScaI digest of pJL062 was used for transformation of B. subtilis HB1122 (19), HB0618 (20), and HB8010 (21) were used for the transduction of CU1065 to give strains containing reporter fusion for perR-FLAG in amyE locus designated HB9620 or HB9735, respectively.

Construction of Reporter Fusion Strains—SPβ phages from HB1122 (19), HB0618 (20), and HB8010 (21) were used for the transduction of CU1065 to give strains containing reporter fusion for mrgA-cat-lacZ, feuA-cat-lacZ, and yciC-cat-lacZ, respectively. The HB9738 strain was made by transduction of HB9735 with SPβ phage from HB1122.

SDS-PAGE Sample Preparation and Analysis—For Fig. 1A, purified PerR protein was incubated with 10 mM DTT, 10 mM H2O2, 10 mM diamide, or 10 mM EDTA at 80 °C for 5 min in SDS sample buffer containing 2% SDS and resolved by non-reducing SDS-PAGE using a Tris-Tricine buffer system (22). For Fig. 1B, purified PerR protein was treated with 1 mM DTT, 10 mM H2O2, or 10 mM diamide at room temperature for 30 min in buffer A, and residual reagents were removed by precipitation and washing with 10% trichloroacetic acid. Recovered samples were modified by 50 mM AMS in the presence of 10 mM EDTA and 2% SDS and separated by non-reducing SDS-PAGE. For Fig. 4B, under anaerobic conditions 10 μM PerR (in 20 μl) was treated with 100 μM Fe2+ or Mn2+ for 5 min and then exposed to varying amounts of H2O2 for 10 min. PerR was recovered by precipitation with 20 μl of 20% trichloroacetic acid. After washing with 10% trichloroacetic acid, the pellets were resuspended in buffer A containing 2% SDS, 20 mM EDTA, and 50 mM AMS and incubated for 30 min in the dark. Then the samples were analyzed by non-reducing SDS-PAGE.

On-gel Zn2+ Detection—PerR protein (20 μg) treated with 10 mM DTT or 10 mM H2O2 was resolved on non-reducing SDS-PAGE gels. The SDS-PAGE gel was soaked in buffer A containing 500 μM PAR for 1 min, and subsequently 50 mM H2O2 was added to release Zn2+. Because the color development (light orange) was transient, a photograph of the gel was taken within 10 min.

Analysis of SDS-PAGE-fractionated Protein by MALDI-TOF MS—To alkylate reduced Cys residues, the SDS-PAGE gels were directly submersed and incubated in buffer A containing 50 mM EDTA and 50 mM iodoacetamide for 30 min with gentle rocking in the dark. Then the gels were stained with Coomassie Brilliant Blue R and subsequently destained. The stained bands were cut and washed three times by incubating with 100 μl of 50% acetonitrile, 50 mM ammonium bicarbonate for 15 min at 37 °C. The washed gel pieces were dried for 20 min in a Speed-Vac and rehydrated with 10–20 μl of trypsin solution (20 ng/μl trypsin in 9% acetonitrile solution containing 40 mM ammonium bicarbonate). The samples were digested for ~4 h (Fig. 2; partial digestion) or ~18 h (Fig. 3; full digestion) at 37 °C. A volume of 0.5 μl of sample was mixed with an equal volume of matrix (saturated solution of α-cyano-4-hydroxytranssuccinic acid in 50% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid) on the target plate and allowed to air dry. MALDI mass spectra were recorded with an Applied Biosystems 4700 mass spectrometer. For the analysis of oxidation of PerR:Zn,Mn (Fig. 5), PerR::Zn,Mn (10 μM purified PerR) was treated with 0, 0.1, 1, 10, or 100 mM H2O2 for 10 min under anaerobic conditions, and the proteins were recovered by precipitation with 10% trichloroacetic acid, modified using 50 mM iodoacetamide, trypsinized, and analyzed using a Bruker BIFLEXIII mass spectrometer.
**Bacillus subtilis PerR Structural Zn²⁺ Site**

**A**

| M | AAEH | EAL | TELK | ETGV | T²TPQ | HAILEY | LNVSMAHPTA | Q4DDIK | T5   |
|---|------|-----|------|------|-------|---------|-------------|--------|------|
| T1 | 668.37 | 603.45 | 561.30 | 614.36 | 2401.19 |
| T2 | 517.30 | 1625.91 | 421.26 | 632.36 | 1086.50 |
| T3 | 1208.88 | 1371.63 | 1485.70 | 1613.79 | 2083.91 |
| T4 | 635.34 | 1139.16 |

**B**

- **T11** (containing ³⁵⁶C) corresponds to the peak of m/z = 1369.66, and T14 (containing ³⁵⁶C) corresponds to the peak of m/z = 2083.91, T14 + T15 corresponds to the peak of m/z = 3199.93. Note that each of these peaks is characterized by a loss of 2 m/z units from the calculated m/z value, indicative of disulfide bond formation between Cys residues.

**C**

**Electrospray Mass Spectrometry Analysis**—Electrospray ionization MS was performed using a Bruker Esquire-LC ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). The ion spray voltage was set at 4 kV, the orifice voltage was set at 80 V, and the interface temperature was set at 80 °C. PerR (stored in buffer A) was buffer-exchanged with 10 mM ammonium bicarbonate (pH 8.0) by using a Micro Bio-Spin 6 chromatography column. For standard acidic denaturing conditions, 25% (v/v) methanol and 1% (v/v) acetic acid in 5 mM ammonium bicarbonate was used as vector solution. Sample volumes of 20 μl were infused into the spectrometer using a syringe pump at a flow rate of 3 μl/min. Mass spectra were analyzed and manipulated using the Bruker Daltonics DataAnalysis program.

**Measurement of Zn²⁺ Release by H₂O₂** Using PAR—Under our buffer conditions (20 mM Tris (pH 8.0), 100 mM NaCl, and 5% glycerol; 25 °C) the absorption maximum of Zn²⁺-PAR complex was observed at 494 nm, and the ε₄₉₄₄₄ nm was measured to be 8.5 ± 0.25 × 10⁴ M⁻¹ cm⁻¹ in the range of 0–6 μM Zn²⁺ (compared with ε₄₉₄₄₄ nm = 6.6 ± 0.2 × 10⁴ M⁻¹ cm⁻¹ for (PAR)₂-Zn²⁺ at pH 7 (23)). 5 μM PerR (PerR:Zn) in buffer A (or buffer A containing 7 M urea) was treated with 0, 0.1, 1, 10, or 100 mM H₂O₂ in the presence of 100 μM PAR at 25 °C, and released Zn²⁺ was measured by monitoring the Zn²⁺-PAR complex at 494 nm every 1 s for 30 min. Because the affinity of PAR to Zn²⁺ was high and the rate of association of the Zn²⁺ with PAR was much faster than that of Zn²⁺ release from PerR (reported value of second order k₁ for forming Zn²⁺-PAR complex at pH 7 is ~2 × 10⁷ M⁻¹ s⁻¹), all the released Zn²⁺ ions were assumed to be present as Zn²⁺-PAR complex. The data were fitted to an effective first order rate equation, A = A₀(1 - e⁻ᵗ/ᵏ[H₂O₂])² + B, where A is absorbance at 494 nm, A₀ is the amplitude of absorbance, k is the second order rate constant, t is time in seconds, and B is the initial absorbance.

**β-Galactosidase Assay**—Overnight cultures of cells in MM containing 10 μM FeCl₃ and 10 μM MnCl₂ were washed with MM and inoculated by 25× dilution into fresh MM containing 10 μM FeCl₃ or 10 μM MnCl₂. At an A₆₅₀ of ~0.6, aliquots of each culture were treated with H₂O₂ and further incubated for 30 min. Alternatively cells were treated with H₂O₂ for 10 min, residual H₂O₂ was removed by centrifugation, and cells were resuspended in fresh MM containing metal ions and further incubated for 30 min. β-Galactosidase assays were performed as reported previously (2).
10 μM FeCl₃ or MM containing 10 μM MnCl₂. At $A_{600} = 0.6$ aliquots of 10-ml culture were mixed with 1.1 ml of trichloroacetic acid at a given time point before and after H₂O₂ treatment. After centrifugation, pellets were resuspended in 10% trichloroacetic acid, split into two fractions, and washed with acetone. Each fraction was sonicated in 150 μl of 20 mM iodoacetamide or 20 mM AMS solution containing 200 mM Tris (pH 8.0), 5% glycerol, 1% SDS, and 20 mM EDTA. Alkylations were performed at room temperature in the dark for 1 h, and ~10 μl (corresponding to 30 μg of protein as assayed by DC protein assay (Bio-Rad)) of alkylated samples were resolved by SDS-PAGE. After electrophoresis, proteins were blotted to a polyvinylidene difluoride membrane and probed with polyclonal anti-FLAG antibody from rabbit and anti-rabbit antibody conjugated with alkaline phosphatase.

Analysis of in Vivo Oxidation of PerR by MS—HB9738 cells were grown overnight in 25 ml of MM containing 10 μM FeCl₃ and 10 μM MnCl₂. Cells were washed twice with MM and inoculated into 0.8 liter of MM supplemented with 10 μM MnCl₂. At an $A_{600}$ of ~0.5, aliquots of 250-ml cell culture were treated with 0, 0.1, or 10 mM H₂O₂ for 5 min, harvested by centrifugation at 6000 rpm for 10 min, and washed twice with Tris-buffered saline (50 mM Tris (pH 7.4) and 150 mM NaCl) containing 20 mM EDTA (TBS + EDTA). Cells were sonicated in 1.3 ml of TBS + EDTA containing protease inhibitor mixture, 1 mg/ml lysozyme, and 2% Triton X-100. After removal of cell debris by centrifugation at 16,100 $g$ of protein as assayed by image 231x26 to 258x38 was increased in intensity by DTT (lane 2). These results suggest that band 1 may contain Zn²⁺ that remains associated with PerR even during electrophoresis in the presence of SDS. Removal of Zn²⁺ from PerR was observed when EDTA was present in the SDS sample buffer (lanes 5 and 6) but not when native protein was treated with EDTA (e.g., during purification).

We used the cysteine-alkylating agent AMS to determine whether all four Cys residues in PerR were reduced. For this experiment, samples were treated with DTT, H₂O₂, or diamide for 30 min in the absence of SDS prior to analysis of AMS reactivity (in the presence of SDS and EDTA to remove associated Zn²⁺). Treatment of purified PerR with AMS resulted in a single band corresponding to the addition of four AMS moieties to PerR (Fig. 1B, lane 2). The appearance of a single modified band despite the presence of two bands in the starting material (Fig. 1A, lane 1) is consistent with the hypothesis that the difference in mobility of bands 1 and 2 is not due to proteolyis but instead reflects the presence or absence of Zn²⁺. Oxidation of Cys residues was apparent after treatment of PerR with 10 mM H₂O₂ for 30 min (as judged by the reduced susceptibility of the protein to AMS modification; lane 6) but not after exposure to diamide (lane 8). Thus, the ability of diamide to oxidize PerR (Fig. 1A, lane 4) requires the presence of harsher conditions (e.g., SDS and heating) relative to H₂O₂. Indeed PerR:Zn,Mn retains DNA binding activity even after treatment with 50 mM diamide for 15 min (data not shown).

These results suggest that band 1 corresponds to monomeric PerR containing bound Zn²⁺, whereas band 2 represents PerR that lacks bound Zn²⁺. This supposition is supported by direct staining of the SDS-PAGE gels with the Zn²⁺-binding dye, PAR. Zn²⁺ was clearly associated with the protein as purified and after DTT treatment but not with the H₂O₂-treated sample (Fig. 1C). Moreover, in the presence of DTT, incubation of PerR with Zn²⁺, but not other divalent cations, greatly increased the fraction of band 1 (data not shown), whereas all four single Cys → Ser mutants migrate in the position of band 2 during SDS-PAGE, suggesting that Zn²⁺ binding has been eliminated (9).

PerR Apoprotein Forms Intrasubunit Disulfide Bonds—We initially speculated that the mobility difference between bands 1 and 2 might originate from an N- or carboxyl-terminal proteolytic cleavage during SDS-PAGE sample preparation. To explore this possibility, we treated an SDS-polyacrylamide gel with iodoacetamide prior to Coomassie Blue staining and destaining (to prevent oxidation of Cys residues) and analyzed the two bands using in-gel tryptic digestion and MALDI-TOF MS analysis. We detected the amino-terminal tryptic peptide (T1) (in Met-excised form) in protein from both bands 1 and 2 implying that there was no difference at the amino terminus.
Bacillus subtilis PerR Structural Zn$^{2+}$ Site

consistent with electrospray ionization MS data of intact protein; see supplemental Fig. 1S). Although the carboxyl-terminal tryptic peptide (T15 and T16 K and ENH) could not be detected under the m/z range of our experiments, we did detect carboxyl-terminal epitope tag in both bands 1 and 2 by immunoblotting analysis of PerR-FLAG (data not shown). These data are consistent with the hypothesis that the primary difference between bands 1 and 2 is the presence of bound Zn$^{2+}$ in band 1.

In the spectrum of PerR isolated from band 2, the tryptic peptides containing $^{96}$CXX$^{99}$ and $^{136}$CXX$^{139}$ (T11, m/z = 2083.91; T14, m/z = 1369.66; and T14 + T15, m/z = 1497.75) were each detected with a loss of 2 m/z units from the calculated values. This mass change is indicative of an intrafragment disulfide bond formation. No peaks corresponding to interfragment disulfide bond formation were detected in this air-oxidized protein. In contrast, peptides from band 1 contained reduced Cys residues as judged by their quantitative modification by iodoacetamide. The modified peptides (labeled as T11*, m/z = 2199.93; T14*, m/z = 1485.70; and T14 + T15*, m/z = 1613.79) display a 114-Da mass increase (57 Da for each carbamidomethyl-modified Cys residue). Thus, PerR in band 1 is fully reduced as expected for a Cys4:Zn$^{2+}$ site, whereas PerR in band 2 is oxidized. In this case, air oxidation leading to the appearance of band 2 likely occurs after unfolding of the protein by SDS because AMS modification (Fig. 1B) and iodoacetamide modification followed by electrospray ionization MS (data not shown) indicate that all four Cys residues are fully reduced in the protein as purified.

**Fig. 3.** Effects of Cys oxidation by H$_2$O$_2$ on the mobility of PerR during SDS-PAGE. A, purified PerR was treated with 0 (lane 1), 10 (lane 2), or 100 mM H$_2$O$_2$ (lane 3) and resolved by SDS-PAGE. Lanes M are protein molecular mass standards. B–D, after treatment of the whole gel with 50 mM iodoacetamide, band 2 (C), and band 4 (D) were analyzed by in-gel tryptic digestion followed by MALDI-TOF MS. Spectra in the upper right insets correspond to the regions of m/z values from 3300 to 3700 with intensities multiplied by 10. E, schematic representations of bands 1, 2, 3, and 4. The dotted line indicates coordination of Zn$^{2+}$ by Cys thiolate (band 1) or disulfide bonds between Cys residues (bands 2–4). For band 4, alternate disulfide bond(s) formation is also possible.
Together these results indicate that the mobility of PerR during SDS-PAGE is a sensitive indicator of conformational changes elicited by Zn$^{2+}$ binding and by disulfide bond formation. Oxidation leading to disulfide bond formation between vicinal Cys residues (in the two CXXC motifs) led to a lower mobility species (band 2) that was further retarded under conditions leading to sulfonic acid formation (band 3). In contrast, the more compact conformation of PerR due to the presence of bound Zn$^{2+}$ (band 1) could be mimicked by one or more disulfide linkages between the two CXXC motifs (band 4). These results are consistent with the previously proposed model of an intrasubunit Cys$_4$:Zn$^{2+}$ site as supported by homology modeling of PerR on the Fur$_{PA}$ structure and by the observation that all four PerR Cys→Ser mutant proteins migrated at a position corresponding to band 2 during SDS-PAGE analysis (supplemental material in Ref. 9).

**Oxidation of PerR Leads to Release of Zn$^{2+}$ Independent of Metal Binding at the Regulatory Site—Oxidation of the Cys$_4$:Zn$^{2+}$ site in the stress-regulated *E. coli* chaperone HSP33 occurs under conditions of severe heat and oxidative stress and activates chaperone activity (24). We reasoned that the Cys$_4$:Zn$^{2+}$ site in PerR might also serve a regulatory role *in vivo*. Specifically under conditions in which PerR exists predominantly in the manganese-cofactored form (PerR:Zn,Mn), oxidation of the Zn$^{2+}$ site might still allow derepression under severe oxidative stress.

To test this hypothesis, we first measured the rate of Zn$^{2+}$ release from PerR:Zn in the presence of H$_2$O$_2$. Release of Zn$^{2+}$, as monitored by formation of the colored PAR complex, was dependent on added H$_2$O$_2$ with a second order rate constant of 0.054 ± 0.009 M$^{-1}$ s$^{-1}$ (Fig. 4A). This corresponds to a half-time of 21.4 min in the presence of 10 mM H$_2$O$_2$. Even in the presence of 7 M urea, the rate of H$_2$O$_2$-mediated Zn$^{2+}$ release was very slow (0.119 ± 0.029 M$^{-1}$ s$^{-1}$; data not shown). These data are consistent with a structural role for the Cys$_4$:Zn$^{2+}$ site that helps maintain a locally folded domain even in the presence of protein denaturants such as urea and SDS.

To monitor oxidation of the Cys$_4$:Zn$^{2+}$ site in the PerR:Zn,Fe and PerR:Zn,Mn forms of the protein we used AMS to monitor the number of reduced cysteines (Fig. 4B). In each case, no significant oxidation of protein was detected by 1 mM H$_2$O$_2$, and ~30% of the protein was fully oxidized by 10 mM H$_2$O$_2$. Complete oxidation of all four Cys residues could be attained.
We also monitored the effects of high levels of H$_2$O$_2$ on reconstituted PerR:Zn,Mn protein by MALDI-TOF MS studies (Fig. 5). Consistent with the AMS modification studies, iodoacetamide modification was reduced in the 10 mM H$_2$O$_2$-treated samples and eliminated in the samples treated with 100 mM H$_2$O$_2$. There was a near quantitative +16-Da mass shift in the two peptides containing Met residues (T5 and T7) but no evidence of significant oxidation of the peptide containing His$_91$ (T11). Thus, PerR:Zn,Mn is insensitive to oxidation in vitro at the Mn$^{2+}$-binding site, even under severe conditions, and is instead inactivated by oxidation of the structural Cys$_{4}$Zn$^{2+}$ site.

**Inactivation of PerR:Zn,Mn Under Severe Oxidative Stress in Vivo Is Not Mediated by Cys Oxidation**—In numerous studies, we have observed that the induction of PerR-regulated genes is highly sensitive to the metal composition of the growth medium. In medium containing iron or iron and manganese, induction is efficient with complete derepression elicited by <100 mM H$_2$O$_2$ or by exposure to NO-generating agents. In contrast, in low iron minimal medium supplemented with manganese, the resulting PerR:Zn,Mn form of the repressor is relatively insensitive to peroxide- and NO-mediated inactivation (2, 10, 25).

To determine whether oxidation of the Cys$_{4}$Zn$^{2+}$ site might contribute to PerR regulation in vivo, we first determined the levels of H$_2$O$_2$ needed to inactivate the PerR:Zn,Mn form of the repressor. Whereas induction in iron-containing minimal medium is readily observed with 0.1 mM H$_2$O$_2$, it took 10 mM H$_2$O$_2$ to induce expression in the manganese-supplemented minimal medium (Fig. 6A). Similar results were observed in a strain expressing PerR-FLAG rather than PerR (data not shown). Although Fur also contains a structural Zn$^{2+}$ ion, we were unable to detect peroxide-mediated inactivation of Fur (Fig. 6A). Similarly there was no evidence for derepression of a reporter fusion (P$_{yciC}$-cat-lacZ) regulated by the third Fur paralog in B. subtilis, Zur (data not shown). The high selectivity of peroxide inactivation is consistent with previous transcriptome analyses: treatment of cells with micromolar levels of H$_2$O$_2$ led to derepression of PerR-regulated genes but not genes controlled by Fur or Zur (10).

Based on our *in vitro* analyses, it seemed likely that the *in vivo* inactivation of PerR:Zn,Mn by 10 mM H$_2$O$_2$ was due to oxidation of Cys residues associated with the structural Zn$^{2+}$ site. To test this hypothesis directly, we used AMS modification to trap reduced Cys residues before and after treatment of cells with 10 mM H$_2$O$_2$. Under all conditions tested (Fig. 6B and data not shown), all four Cys residues were fully modified by AMS. In some experiments, there was a faint band corresponding to unmodified PerR after treatment, consistent with formation of either disulfide or sulfonic acid modifications (data not shown). However, this band was always a minor species, and it seemed unlikely that this small amount of oxidation was sufficient to account for the observed derepression (Fig. 6A). These data led us to reject the hypothesis that inactivation of PerR *in vivo*, in the presence of 10 mM H$_2$O$_2$, is principally mediated by Cys oxidation.

---

**FIGURE 5. Oxidation of PerR:Zn,Mn by H$_2$O$_2$ leads to disulfide bond formation between Cys residues without His modification.** PerR:Zn,Mn (10 mM purified PerR + 100 mM MnCl$_2$) was incubated with 0 (A), 0.1 (B), 1 (C), 10 (D), or 100 (E) mM H$_2$O$_2$ for 10 min under anaerobic conditions. Proteins were recovered by precipitation with 10% trichloroacetic acid, modified using 50 mM iodoacetamide, trypsinized, and analyzed by MALDI-TOF MS. Peptides containing two carbamidomethyl-modified Cys residues are designated with open triangles and marked with asterisks as in Fig. 2. CXXC motif-containing peptides (T11 and T14) are exclusively detected as fully alkylated forms up to 1 mM H$_2$O$_2$ treatment. In samples treated with 100 mM H$_2$O$_2$ (E), CXXC motif-containing peptides are detected without alkylation at Cys residues. Note that T11 shows no increase in oxidation at His$_{91}$ residue despite the full oxidation of Cys$_{4}$ and Cys$_{99}$ residues by 100 mM H$_2$O$_2$ treatment (T11 contains a small amount of oxidation as judged by the small peak with a +16-Da mass shift. This oxidation is present in the purified PerR protein and is not further increased by peroxide treatment of Mn$^{2+}$-supplemented protein. This corresponds to His$_{91}$ oxidation and correlates with the observation that the protein, as purified, is approximately 60% active for DNA binding.) T7 containing Met$_{54}$ gains +16 m/z units, indicative of methionine sulfoxide formation. By analogy, the gain of +16 m/z units on T5 seems to indicate the oxidation at Met$_{54}$ rather than His$_{91}$.

with 100 mM H$_2$O$_2$. These results suggest that the rate of oxidation of the Cys$_{4}$Zn$^{2+}$ site is similar in the PerR:Zn (Fig. 1B, lane 6), PerR:Zn,Fe, and PerR:Zn,Mn forms of the repressor (Fig. 4B). The rate of oxidative inactivation measured here also explains the previously reported 50% inactivation of DNA binding activity noted for reconstituted PerR:Zn,Mn by treatment with 10 mM H$_2$O$_2$ for 20 min (9). In contrast, the DNA binding activity of the PerR:Zn,Fe form is highly sensitive to peroxide inactivation (estimated $k_{\text{inact}} \sim 10^{5}$ M$^{-1}$ s$^{-1}$) due to histidine oxidation at the Fe$^{2+}$-binding site (9).
In Vivo Oxidation of PerR:Zn,Mn Is Correlated with Regulatory Site Oxidation—To determine what modification(s) might be associated with PerR:Zn,Mn inactivation in vivo, we immunoprecipitated FLAG epitope-tagged PerR from B. subtilis cells after treatment with either 0.1 or 10 mM H$_2$O$_2$ (Fig. 7A). MALDI-TOF analysis of in situ trypsin-digested PerR was consistent with the presence of four reduced Cys residues under all conditions: in each case the T11 and T14 tryptic peptides were fully alkylated by iodoacetamide. In the sample treated with 10 mM H$_2$O$_2$, the T11 peptide appeared as a doublet with ~30–40% oxidation corresponding to a species previously shown to represent His$^{91}$ oxidation (9). The other histidine residue previously shown to be oxidized by Fe$^{2+}$-mediated hydroxyl radical modification is in peptide T5. This peptide appeared as a doublet (with a 16-Da mass shift) in all three samples, likely due to methionine sulfoxide formation during sample preparation and analysis (Met oxidation is also consistent with the doublet noted for T7, the other Met-containing peptide). With this background oxidation and the poor recovery of highly oxidized T5 peptides noted during matrix desorption, it is difficult to assess the level of His$^{91}$ oxidation. Nevertheless these results indicate that the in vivo oxidation of PerR:Zn,Mn leads to oxidation of His$^{91}$ in the regulatory metal-binding site but not to significant Cys oxidation.

Treatment of reconstituted PerR:Zn,Mn (in the absence of Fe$^{2+}$) with 10–100 mM H$_2$O$_2$ oxidized the Cys$_4$:Zn$^{2+}$ site but did not lead to appreciable His oxidation (Fig. 5). In contrast, in vivo oxidation in cells containing PerR:Zn,Fe led to His$^{91}$ oxidation with no significant Cys oxidation. Because this pattern of oxidation is characteristic of PerR:Zn,Fe (9), we suggest that treatment of cells with 10 mM H$_2$O$_2$ led to the release of sufficient intracellular Fe$^{2+}$ to allow a shift in the active species of PerR from PerR:Zn,Mn to PerR:Zn,Fe.

PerR:Zn Binds Fe$^{2+}$ with Higher Affinity than Mn$^{2+}$—We next determined the affinity of PerR:Zn for Fe$^{2+}$ and Mn$^{2+}$...
using a fluorescence anisotropy-based DNA binding assay (Fig. 8). The apparent $K_d$ for activation by Fe$^{2+}$ (~0.1 μM) is significantly greater than that for Mn$^{2+}$ (~2.8 μM). Although lower absolute affinities were observed in several replicate experiments (particularly for Fe$^{2+}$, perhaps due to trace oxygen contamination), the data are generally consistent with a significant preference for Fe$^{2+}$ relative to Mn$^{2+}$. Indeed we previously demonstrated that 10 μM Mn$^{2+}$ is unable to protect PerR:Zn against oxidative inactivation in the presence of 10 μM Fe$^{2+}$, and only partial protection is afforded by 100 μM Mn$^{2+}$ (9). We also estimated the affinity for Mn$^{2+}$ using electrophoretic mobility shift assay experiments. By inclusion of Mn$^{2+}$ in the binding and running buffers as well as in the polyacrylamide gel, we could measure the apparent DNA binding affinity of PerR as a function of Mn$^{2+}$ concentration (supplemental Fig. S2). Under these assay conditions, DNA binding affinity increased 40-fold as the Mn$^{2+}$ concentration was increased from 1 to 100 μM. Together these studies indicate that PerR:Zn is activated for DNA binding by Mn$^{2+}$ in the low micromolar range and by Fe$^{2+}$ with even higher affinity.

**DISCUSSION**

Cells respond to oxidative stress by the inducible synthesis of a variety of protective enzymes and proteins. Most peroxide sensors characterized to date use cysteine thiolates to detect peroxides, often leading to the formation of either intra- or intermolecular disulfides (12–14). In contrast, *B. subtilis* PerR has been shown recently to detect low levels (<100 μM) of H$_2$O$_2$ by iron-catalyzed oxidation of either of two His residues, His$^{37}$ and His$^{91}$ (9). These two His residues are required for Fe$^{2+}$ binding and are predicted, by homology modeling, to be direct ligands to the regulatory metal ion. PerR also contains a tightly bound Zn$^{2+}$ ion (1) proposed to exist as part of a Cys$_4$Zn$^{2+}$ site (9). In the present study we investigated the redox sensitivity of this Cys$_4$Zn$^{2+}$ site and assessed the role of this site in sensing high levels of H$_2$O$_2$ both *in vitro* and *in vivo*.

PerR retained tightly bound Zn$^{2+}$ even after purification in the presence of EDTA. In the course of our studies, we noted a correlation between conditions favoring Zn$^{2+}$ binding and the appearance of a more rapidly migrating form of PerR during SDS-PAGE (band 1). Using MALDI-TOF analysis of *in situ* digested protein samples, we demonstrated that non-reducing SDS-PAGE provides a sensitive assay for monitoring the status of the Cys$_4$Zn$^{2+}$ site. When this site was intact, PerR migrated as a 14.5-kDa protein, whereas when Zn$^{2+}$ was lost the resulting protein migrated at the expected position of 16.4 kDa. Loss of Zn$^{2+}$ was accompanied by oxidation of PerR to a disulfide-bonded form. When treated with high levels of H$_2$O$_2$ (in the presence of protein denaturant and heat) two additional bands appeared including a slowly migrating band in which PerR contains two sulfonic acid residues and one disulfide bond (band 3) and a more rapidly migrating band containing one or two long range disulfide bonds (band 4).

Because many Fur homologs are known or predicted to contain a structural Zn$^{2+}$ site, our results may provide an explanation for the observation that many Fur homologs migrate as a doublet during SDS-PAGE. Examples include *B. subtilis* and *Bacillus cereus* Fur (26, 27), *Streptomyces reticuli* FurS (15), and *Streptomyces coelicolor* CatR (16). However, analysis of *E. coli* Fur by amino-terminal sequencing and by electrospray ionization MS revealed that the fast migrating band is a proteolytic product of intact protein lacking the amino-terminal nine amino acids (28, 29). Recently it has been noted that Zn$^{2+}$ binding is required for stable folding of the carboxyl-terminal domain of *E. coli* Fur and for protein dimerization (30).

Previously we (1) and others (15, 16) had suggested that PerR and its homologs might sense H$_2$O$_2$ by Cys-based redox reactions. However, each of these studies used high levels of H$_2$O$_2$ (5–10 mM), and the physiological role, if any, of this Cys-based oxidation has not been established. In the present study we demonstrated that the structural Cys$_4$Zn$^{2+}$ site in PerR was remarkably resistant to H$_2$O$_2$ with an observed second order rate constant for Zn$^{2+}$ release of ~0.054 M$^{-1}$ s$^{-1}$ (corresponding to a half-time for inactivation of ~20 min by 10 mM H$_2$O$_2$). A similar oxidation rate was determined by several assays including Zn$^{2+}$ release studies (using PerR:Zn; Fig. 4A), monitoring of Cys status by AMS modification of PerR:Zn,Fe and PerR:Zn,Mn (Fig. 4B), and monitoring of Cys status by iodacetamide modification of PerR:Zn,Mn followed by MALDI-TOF MS (Fig. 5) and by measuring oxidative inactivation of DNA binding activity for PerR:Zn,Mn (9). Significantly the Cys$_4$:Zn$^{2+}$ site in PerR was even less reactive than free cysteine (2–20 mM$^{-1}$ s$^{-1}$; Refs. 31–33), the major low molecular weight thiol in *B. subtilis* (34). Thus, these Cys residues are actually protected against peroxidative attack by Zn$^{2+}$ coordination. Comparison of the rate reported here for oxidation of the Cys$_4$:Zn$^{2+}$ site ($k_{\text{inact}}$ ~ 0.05 M$^{-1}$ s$^{-1}$) with the rate of peroxide-mediated inactivation of PerR:Zn,Fe ($k_{\text{inact}}$ ~ 10$^6$ M$^{-1}$ s$^{-1}$) highlights the fact...
that the structural Zn\(^{2+}\) and regulatory Fe\(^{2+}\) sites have vastly different sensitivities toward oxidation.

The oxidative modification of thiolates coordinated to Zn\(^{2+}\) has been implicated in several other redox-sensitive proteins. The \textit{S. coelicolor} RsrA anti-\(\sigma\) factor is regulated by disulfide stress. Treatment with diamide, a thiol-specific oxidant, leads to Zn\(^{2+}\) release and inactivation of the anti-\(\sigma\). This results in activation of \(\sigma^8\) and expression of thiol reductants such as thioredoxin and thioredoxin reductase (35, 36). \textit{E. coli} HSP33 is a redox-regulated protein chaperone that contains a high affinity Cys\(_4\)Zn\(^{2+}\) site (17). Oxidation of this Cys\(_4\)Zn\(^{2+}\) site, accompanied by Zn\(^{2+}\) release, activates this protein chaperone, which can functionally replace the redox-sensitive DnaK protein under severe oxidative stress conditions (24). Activation of HSP33 requires harsh conditions including high levels of peroxides (e.g. 4 mM \(\text{H}_2\text{O}_2\)) and elevated temperatures (e.g. 43 °C). \textit{B. subtilis} also contains an HSP33 ortholog (37), but the redox regulation of this protein has not yet been characterized.

Because the Cys\(_4\)Zn\(^{2+}\) site in PerR is structurally similar to the regulatory site in HSP33, we speculated that oxidation at this metal center might provide a backup mechanism for induction under severe oxidative stress conditions for iron-starved cells (in which PerR would be present in the PerR:Zn,Mn form). Indeed \textit{in vitro} oxidation of reconstituted PerR:Zn,Mn by 10 or 100 mM \(\text{H}_2\text{O}_2\) was correlated with Cys oxidation and not with oxidation of His residues in the regulatory metal-binding site (Fig. 5). In contrast, \textit{in vivo} treatment of manganese-supplemented cells with 10 mM \(\text{H}_2\text{O}_2\) (a level sufficient to induce a PerR-regulated reporter; Fig. 6) did not lead to PerR Cys oxidation (Fig. 7). The lack of Cys oxidation \textit{in vivo} may be explained by the lower effective concentration of \(\text{H}_2\text{O}_2\) \textit{in vivo} due to the presence of catalase, peroxidases, and competition from low molecular mass thiols such as cysteine. Instead induction was correlated with oxidation of at least one of the two His residues previously implicated in peroxide sensing (9). Oxidation of these His residues is diagnostic for PerR:Zn,Fe. It is likely that exposure of cells to high levels of peroxide releases sufficient Fe\(^{2+}\) to allow PerR to switch from the relatively insensitive PerR:Zn,Mn form to the thermodynamically preferred and much more peroxide-sensitive PerR:Zn,Fe form.

To evaluate this model it is necessary to determine (i) the relative affinity of PerR:Zn for Fe\(^{2+}\) and Mn\(^{2+}\) and (ii) the levels of free metal ions in the cytosol under different growth conditions. PerR:Zn binds Fe\(^{2+}\) with higher affinity than Mn\(^{2+}\) as judged by both metal-dependent DNA binding measurement (Fig. 8) and the ability of Mn\(^{2+}\) to protect against Fe\(^{2+}\)-catalyzed protein inactivation (9). This is consistent with the observation that PerR:Zn only forms in cells grown under iron-limiting conditions. Although it is difficult to measure the levels of free metal ions in the cell, it is possible to make reasonable estimates. For example MntR, the \textit{B. subtilis} protein responsible for regulating Mn\(^{2+}\) levels in the cell binds two Mn\(^{2+}\) per monomer with apparent dissociation constants (at pH 8.0) of 0.2–2.0 and 5–13 \(\mu\)M (38). Somewhat lower affinities were reported based on EPR measurements of Mn\(^{2+}\) binding by the \textit{Bacillus anthracis} MntR ortholog with average dissociation constants in the range of \(\sim 50 \mu\)M (39). Because MntR represses Mn\(^{2+}\) uptake when in its Mn\(^{2+}\)-bound state, it is reasonable to assume that intracellular levels of free Mn\(^{2+}\) are normally maintained at levels sufficient to saturate PerR (to generate the PerR:Zn,Mn form of the repressor). The levels of exchangeable Fe\(^{2+}\) in the cytosol are difficult to measure directly, but in \textit{E. coli} chelatable iron levels of 10–30 \(\mu\)M have been measured by EPR in the presence of desferrioxamine (40, 41). If similar values pertain to \textit{B. subtilis}, this would explain why, in the presence of both iron and manganese in the growth medium, PerR is predominantly in the peroxide-sensing PerR:Zn,Fe form. Apparently in cells grown in manganese-supplemented minimal medium (with no added iron), the intracellular level of iron is lowered sufficiently to allow the PerR:Zn,Mn form of the repressor to predominate (2, 25). Yet because of the relatively higher affinity for Fe\(^{2+}\), a small increase in intracellular free Fe\(^{2+}\) could shift the repressor from the PerR:Zn,Mn to the PerR:Zn,Fe form. Treatment of cells with 10 mM \(\text{H}_2\text{O}_2\) is likely to damage iron-sulfur clusters (42) and perhaps other non-heme iron proteins, leading to elevation of free Fe\(^{2+}\) in the cell.
Bacillus subtilis PerR Structural Zn$^{2+}$ Site

25. Moore, C. M., Nakano, M. M., Wang, T., Ye, R. W., and Helmann, J. D. (2004) J. Bacteriol. 186, 4655–4664
26. Bsat, N., and Helmann, J. D. (1999) J. Bacteriol. 181, 4299–4307
27. Harvie, D. R., and Ellar, D. J. (2005) Curr. Microbiol. 50, 246–250
28. Michaud-Soret, I., Adrait, A., Jaquinod, M., Forest, E., Touati, D., and Latour, J. M. (1997) FEBS Lett. 413, 473–476
29. Coy, M., and Neilands, J. B. (1991) Biochemistry 30, 8201–8210
30. Pecqueur, L., D’Autreaux, B., Dupuy, J., Nicolet, Y., Jacquamet, L., Brutscher, B., Michaud-Soret, I., and Bersch, B. (May 11, 2006) J. Biol. Chem. 10.1074/jbc.M601278200
31. Stone, J. R. (2004) Arch. Biochem. Biophys. 422, 119–124
32. Winterbourn, C. C., and Metodiewa, D. (1999) Free Radic. Biol. Med. 27, 322–328
33. Inlay, J. A. (2003) Annu. Rev. Microbiol. 57, 395–418
34. Newton, G. L., Arnold, K., Price, M. S., Sherrill, C., Delcardayre, S. B., Aharonowitz, Y., Cohen, G., Davies, J., Fahey, R. C., and Davis, C. (1996) J. Bacteriol. 178, 1990–1995
35. Kang, J. G., Paget, M. S., Seok, Y. J., Hahn, M. Y., Bae, J. B., Hahn, J. S., Kleanthous, C., Buttner, M. J., and Roe, J. H. (1999) EMBO J. 18, 4292–4298
36. Bae, J. B., Park, J. H., Hahn, M. Y., Kim, M. S., and Roe, J. H. (2004) J. Mol. Biol. 335, 425–435
37. Janda, I., Devedjiev, Y., Derewenda, U., Dauter, Z., Bielnicki, J., Cooper, D. R., Graf, P. C., Joachimiak, A., Jakob, U., and Derewenda, Z. S. (2004) Structure (Lond.) 12, 1901–1907
38. Kliegman, J. J., Griner, S. L., Helmann, J. D., Brennan, R. G., and Glasfeld, A. (2006) Biochemistry 45, 3493–3505
39. Sen, K. I., Sienkiewicz, A., Love, J. F., Vanderspek, J. C., Fajer, P. G., and Logan, T. M. (2006) Biochemistry 45, 4295–4303
40. Keyer, K., and Inlay, J. A. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 13635–13640
41. Woodmansee, A. N., and Inlay, J. A. (2002) Methods Enzymol. 349, 3–9
42. Inlay, J. A. (2006) Mol. Microbiol. 59, 1073–1082