Molecular Microbiology (2008) 68(4), 978–986 ■
doi:10.1111/j.1365-2958.2008.06200.x
First published online 8 April 2008

Oxidant-dependent switching between reversible and sacrificial oxidation pathways for *Bacillus subtilis* OhrR

Sumarin Soonsanga,[1†] Jin-Won Lee[1,2] and John D. Helmann[1*]
[1]*Department of Microbiology, Cornell University, Ithaca, NY 14853-8101, USA.*
[2]*Department of Life Science, Hanyang University, Seoul, Korea.*

Summary

The *Bacillus subtilis* OhrR protein functions as a transcriptional repressor of the inducible peroxidase, OhrA. Derepression is mediated by the organic-peroxide selective oxidation of an active site cysteine (C15). In the presence of cumene hydroperoxide (CHP), oxidation of OhrR leads to a sulphenic acid intermediate which reacts to form either a mixed-disulphide or a protein sulphenamide. These inactive forms of OhrR can be reactivated by thiol–disulphide exchange reactions allowing restoration of repression. Here, we demonstrate that linoleic acid hydroperoxide (LHP) is a potent oxidant for OhrR and even low levels lead to overoxidation of OhrR to cysteine sulphinic (and sulphonic) acid derivatives. Kinetic competition experiments indicate that further oxidation of the initial OhrR sulphenate product occurs at least 100-fold more rapidly with LHP than with CHP. Thus, depending on the oxidant, OhrR can be either reversibly oxidized or can instead function as a sacrificial regulator.

Introduction

Cells exposed to molecular oxygen generate reactive oxygen species (ROS) by the autooxidation of reduced enzyme cofactors and from the incomplete reduction of oxygen during respiration (Imlay, 2003). Cells may also encounter high levels of ROS when exposed to oxidant-generating defence mechanisms such as those found in both animals and plants (Fang, 2004; Torres and Dangl, 2005). Typically, exposure of cells to low levels of ROS leads to the upregulation of appropriate defensive pathways including detoxification enzymes such as superoxide dismutase, catalase and peroxidases.

We have focused our attention on the pathways by which *Bacillus subtilis* senses and responds to peroxides (Helmann *et al*., 2003). The adaptive response to hydrogen peroxide is co-ordinated by the PerR transcription factor, whereas the adaptive response to organic peroxides is controlled by OhrR (Mongkolsuk and Helmann, 2002). The PerR regulon includes the major vegetative catalase (KatA), alkyl hydroperoxide reductase (AhpCF), a Dps-like miniferritin (MrgA), the heme biosynthesis operon, a Zn(II)-transporting P-type ATPase (ZosA), the major regulator of iron homeostasis (Fur) and PerR itself (Chen *et al*., 1995; Herbig and Helmann, 2001; Fuangthong *et al*., 2002; Gaballa and Helmann, 2002). In contrast, OhrR regulates a single gene: the OhrA cysteine-based peroxidase (Fuangthong *et al*., 2001).

PerR is a metal-containing repressor protein, related to ferric uptake regulator (Fur) family of proteins, and binds Fe(II) as a corepressor under most growth conditions (Lee and Helmann, 2007). Upon exposure of cells to H_2O_2, oxidation of the Fe(II) centre in PerR leads to hydroxyl radical generation (Fenton reaction) and this leads to the oxidation of either of two His residues that co-ordinate the metal cofactor (Lee and Helmann, 2006). The oxidized PerR protein is inactive as a repressor and, as far as is currently known, there is no repair pathway for oxidized His residues. Thus, PerR is thought to function as sacrificial regulator.

OhrR is more typical of peroxide-sensing regulators in that it relies on an active site Cys residue (Kiley and Storz, 2004; Jacob *et al*., 2006). The initial step in the inactivation of the OhrR repressor is the oxidation of the C15 thiolate to a sulphenic acid (Fuangthong and Helmann, 2002). Although originally thought to be sufficient for derepression, recent evidence indicates that the OhrR C15 sulphenate (SOH) is still active for binding DNA: loss of activity is correlated with further processing of the initially formed sulphenate to either a mixed disulphide or by cyclization to the protein sulphenamide (Lee *et al*., 2007). This reaction pathway has been documented in a reconstituted *in vitro* system using cysteine as a model low-molecular-weight (LMW) thiol and cumene hydroperoxide (CHP) as a model organic peroxide oxidant. Thiol–disulphide exchange

Accepted 3 March, 2008. *For correspondence. E-mail jdh9@cornell.edu; Tel. (+1) 607 255 6570; Fax (+1) 607 255 3904. †Present address: Microbial Engineering Laboratory, National Center for Genetic Engineering and Biotechnology, 113 Thailand Science Park, Phahonyothin Road, Klong 1, Klong Luang, Pathumthani 12120, Thailand (E-mail: sumarin.soo@biotec.or.th).

reactions can rapidly regenerate active OhrR from the S-thiolated derivatives and slowly regenerate active OhrR from the cyclic sulphenamide (Lee *et al*., 2007). In cells treated with CHP, OhrR also accumulates as mixed disulphide species or as the protein sulphenamide. The major S-thiolated species are the mixed disulphides with a structurally uncharacterized 398 Da LMW thiol, cysteine and coenzyme A. This reaction pathway differs from that documented for those OhrR proteins, such as that from *Xanthomonas campestris*, that additionally contain one or more additional Cys residues in their carboxyl-terminal region (Panmanee *et al*., 2002). In this case, oxidation of OhrR leads to a reversibly inactived form containing an intersubunit disulphide (Panmanee *et al*., 2006; Newberry *et al*., 2007).

Here, we have investigated the reaction of OhrR with linoleic acid hydroperoxide (LHP) which is typical of the types of oxidized fatty acids that are present in the soil environment when there is decaying plant matter. Unexpectedly, the relevant oxidation pathway for the inactivation of OhrR in the presence of LHP differed significantly from that observed with CHP: overoxidation to the sulphinic acid occurred rapidly and thereby inhibited both S-thiolation and cyclic sulphenamide formation. Thus, CHP oxidizes OhrR by a reversible pathway, whereas in the presence of LHP OhrR appears to function as a sacrificial regulator.

Results and discussion

Overview of OhrR oxidation by CHP

Previous studies using CHP as an oxidant (Lee *et al*., 2007) have demonstrated a branched pathway for the functional inactivation of OhrR (Fig. 1). OhrR is initially oxidized to the sulphenic acid (reaction 1), a form still active for binding DNA. This sulphenate can enter any of three different reaction pathways, depending on the concentration of oxidant and the presence of LMW thiols such as cysteine.

In the first pathway (reaction 2) the sulphenic acid is rapidly trapped as a mixed-disulphide with cysteine or other LMW thiols. In the presence of saturating amounts of cysteine (1 mM), the inactivation of OhrR by CHP occurs rapidly with an observed k_{inact} ~2×10^4 M^{-1} s^{-1}. Under these conditions, $k_{inact} \sim k_1$ (Lee *et al*., 2007). This S-cysteinylated protein is reduced by thiol reductants to restore DNA binding activity (reaction 3). The concentration of cysteine in *B. subtilis* is estimated as 0.15–0.5 mM (Newton *et al*., 1996; Gusarov and Nudler, 2005), more than sufficient to mediate efficient S-cysteinylation in the presence of CHP (Lee *et al*., 2007).

In the second pathway, as observed *in vitro* in the absence of cysteine (Lee *et al*., 2007), the initially formed

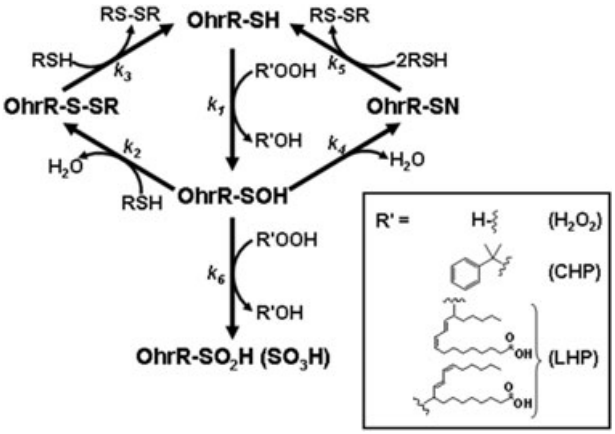

Fig. 1. Oxidation pathways for *Bacillus subtilis* OhrR. OhrR first is oxidized to the sulphenic acid form (reaction 1) which retains DNA binding activity (Lee *et al*., 2007). The OhrR sulphenate is functionally inactivated by either S-thiolation (reaction 2), protein sulphenamide formation (reaction 4), or overoxidation to the sulphinic and sulphonic acid derivatives (reaction 6). The OhrR mixed disulphide can be rapidly re-activated by disulphide exchange in the presence of reduced thiols such as DTT (reaction 3). The OhrR sulphenamide is slowly re-reduced by DTT (reaction 5) whereas the OhrR sulphinic and sulphonic acid derivatives are not.

sulphenic acid slowly cyclizes into the protein sulphenamide which is inactive for DNA binding (reaction 4). Protein sulphenamides form when the cysteine sulphenate (SOH) condenses with a backbone amide group (NH) to generate a cyclic sulphenamide (SN). Protein sulphenamide formation was first described in structural studies of protein tyrosine phosphatase 1B in which the active site Cys215 residue forms a sulphenate that subsequently cyclizes by condensation with the amide group of the adjacent amino acid (Ser216) (Salmeen *et al*., 2003; van Montfort *et al*., 2003). Protein sulphenamides are slowly reduced to an active form by DTT (reaction 5) as shown for both OhrR (Lee *et al*., 2007) and a murine protein tyrosine phosphatase (Yang *et al*., 2007).

In the third pathway, the sulphenic acid can be further oxidized to form the sulphinic (SO_2H) and sulphonic (SO_3H) acid derivatives (reaction 6). These overoxidized forms can not be reactivated by DTT. Previously, overoxidation was observed in cells treated with high levels of CHP or in cells in which LMW thiols were depleted (by addition of the thiol oxidant diamide) prior to exposure to CHP (Lee *et al*., 2007).

Linoleic acid hydroperoxide is a preferred substrate for oxidation of OhrR

Here, we have extended our *in vivo* and *in vitro* analysis of OhrR oxidation to the reaction with LHP. Lipid hydroperoxides are thought to be the physiologically relevant substrate for organic hydroperoxide reductases (Ohr per-

oxidases) present in soil bacteria. Oxidized polyunsaturated lipids from decaying plant material are prevalent in the soil environment and, in *Xanthomonas campestris*, it has been reported that OhrR is highly sensitive to inactivation by LHP (Klomsiri *et al*., 2005). Optimal induction of the OhrR regulated reporter fusion, P_{ohrA}-*cat–lacZ*, requires 100 μM CHP (Fig. 2A), consistent with previous analyses (Fuangthong *et al*., 2001). In contrast, a comparable level of derepression was observed with only 5 μM LHP (Fig. 2A). Thus, LHP is ~20-fold more potent an inducer than CHP *in vivo*. At higher concentrations of LHP (10 and 20 μM) expression of the P_{ohrA}-*cat–lacZ* reporter fusion decreased, perhaps owing to toxicity. The higher relative potency of LHP could be due to increased penetration into cells, a more selective interaction with OhrR, or an increased rate of protein oxidation.

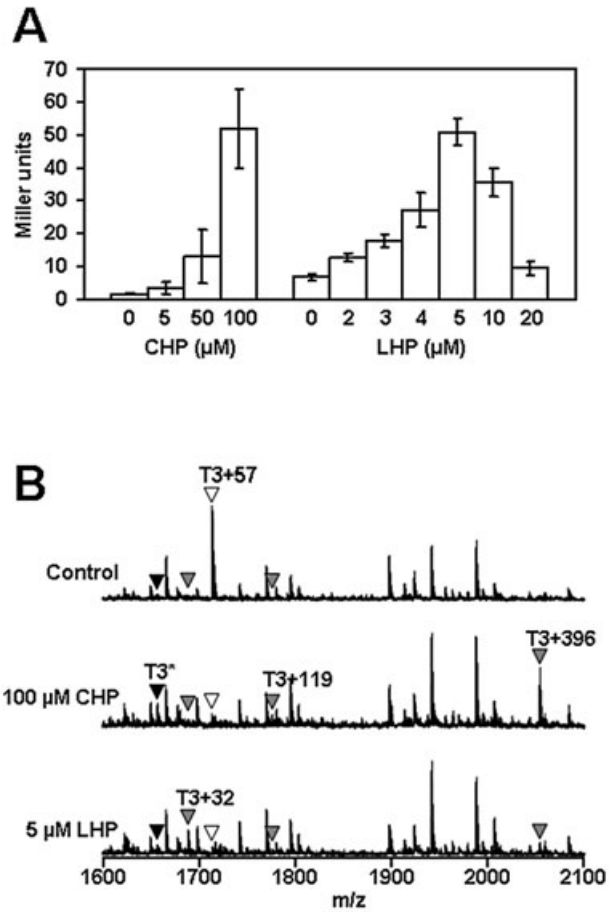

Fig. 2. Overoxidation of OhrR by LHP *in vivo*.
A. Activity of a P_{ohrA}-*cat–lacZ* transcriptional fusion upon oxidation by CHP and LHP. β-Galactosidase activity was measured after 15 min of treatment of mid-logarithmic phase cells with CHP or LHP added at the indicated concentrations. Error bars represent the standard deviation from three independent experiments.
B. MALDI-TOF analysis of OhrR oxidation state in cells treated with either CHP or LHP. Cells were either untreated (top panel) or treated with 100 μM CHP (middle panel) or 5 μM LHP (bottom panel) for 1 min. After alkylation of un-reacted Cys residues with IA, immunoprecipitated and purified OhrR-FLAG was digested with trypsin prior to MALDI-TOF MS analysis. T3 is the Cys15 containing peptide. In the absence of oxidant, C15 is fully alkylated by IA (yielding the T3 + 57 tryptic peptide; white triangle). After exposure to CHP, the T3 peptide now appears as a mixture of three peaks representing the sulphenamide (T3*; black triangle) and the mixed disulphides formed with cysteine (T3 + 119) and a 398 Da thiol (T3 + 396), as previously documented (Lee *et al*., 2007). In contrast, in the LHP-treated cells these products are either not detected or are present at greatly reduced levels. Instead, a prominent peak (grey triangle) corresponding to sulphinic acid formation (T3 + 32) appears.

LHP leads to overoxidation of OhrR in vivo

MALDI-TOF MS analyses were conducted to determine the *in vivo* fate of OhrR in cells treated with LHP (Fig. 2B). For this analysis, cells expressing OhrR-FLAG (from its native promoter) were treated with oxidant, unreacted thiols were alkylated with iodoacetamide (IA), and the OhrR-FLAG protein was recovered by immunoprecipitation. After isolation by SDS-PAGE, the protein was trypsinized and the oxidation state of Cys15 monitored by MALDI-TOF MS.

In untreated samples, OhrR was present as the reduced form and was quantitatively alkylated by IA (detected as a T3 + 57 tryptic peptide peak; T3 corresponds to the peptide from Leu-10 to Arg-23). In the sample treated with 100 μM CHP, we detected a major peak corresponding to S-thiolated protein modified with a 398 Da thiol (T3 + 396), the cyclic sulphenamide (T3*), and a small peak of corresponding to protein S-cysteinylation (T3 + 119), as previously documented (Lee *et al*., 2007). Surprisingly, in the sample treated with 5 μM LHP, the peaks corresponding to S-thiolation and sulphenamide formation were absent or greatly decreased. As there was no residual T3 + 57 peak, we infer that OhrR was quantitatively oxidized to a form non-reactive with IA under these conditions. We do note a prominent T3 + 32 peak, which corresponds to the sulphinic acid derivative of OhrR. Although the peak intensity is lower than the alkylated T3 + 57 peak in the control sample, negatively charged peptides are often poorly detected in positive ion mode MALDI experiments. Indeed, we previously demonstrated that treatment of cells with CHP, after first depleting intracellular thiols with diamide, also led to OhrR overoxidation as detected as a small T3 + 32 peak (and confirmed by ESI-MS). Moreover, OhrR overoxidized to the sulphonic acid is detected in negative ion, but not in positive ion mode (Lee *et al*., 2007). Therefore, we suggest that the major *in vivo* products of LHP oxidation under these conditions are irreversibly overoxidized (e.g. sulphinic and sulphonic acids), rather than reversibly modified, OhrR. Indeed, OhrR oxidized *in vitro* with low concentrations of LHP (150 or 300 nM) incorporates either two or three additional oxygen atoms per monomer as monitored by ESI-MS of

the intact protein, consistent with rapid overoxidation of Cys15 (data not shown).

The kinetics of OhrR inactivation by LHP and CHP differ significantly

The kinetics of OhrR inactivation were further investigated using a fluorescence anisotropy (FA)-based DNA-binding assay. First, OhrR–DNA complexes were treated with various concentrations of either CHP or LHP in the absence of free cysteine (Fig. 3). At one molar equivalent (per protein monomer) of oxidant (300 nM), the rates of OhrR inactivation by CHP and LHP were indistinguishable. This slow rate of inactivation corresponds precisely to the previously determined rate for sulphenamide formation (Lee *et al.*, 2007). We surmise that in these reactions OhrR was initially oxidized to the sulphenic acid, and then DNA dissociation was limited by the relatively slow rate of cyclization to the sulphenamide (Lee *et al.*, 2007). This also indicates that the rate of initial oxidation (to the sulphenate) is faster than the subsequent rate of overoxidation (to the sulphinic and sulphonic acids) for both CHP and LHP. This can be summarized as $k_1 > k_6$ (0.3 μM oxidant). If k_6 were faster than k_1 then treatment with one molar equivalent of oxidant would instead lead to a mixture comprised predominantly of unoxidized OhrR and the sulphinic acid derivative.

Surprisingly, only two molar equivalents of LHP (600 nM) increased the dissociation rate of OhrR from DNA significantly, whereas 600 nM CHP led to essentially the same DNA dissociation rate as treatment with 300 nM CHP (Fig. 3B versus Fig. 3A). As the rate of sulphenamide formation from the sulphenate (k_4) is likely to be the same regardless of the oxidant, we hypothesized that the second molar equivalent of LHP (~300 nM after the initial oxidation event) reacted rapidly with the initially formed OhrR sulphenate resulting in OhrR sulphinic acid. Indeed, protein overoxidation is detected under these conditions by ESI-MS analysis (data not shown). In contrast, overoxidation by CHP required significantly higher levels of oxidant (Fig. 3A). In kinetic terms, we infer that k_6 (0.3 μM LHP) > k_4 > k_6 (0.3 μM CHP). This speculation is confirmed in the following experiments. Thus, depending on the oxidant, the pathway for the functional inactivation of OhrR can change.

Rapid LHP-mediated overoxidation of OhrR to the sulphinic acid in vitro

As previously observed with CHP, OhrR loses DNA binding activity upon formation of S-thiolated protein (reaction 2), the cyclic sulphenamide (reaction 4), or overoxidation (reaction 6) (Lee *et al.*, 2007). These forms of oxidized OhrR species can be distinguished in an FA-based DNA-binding assay based on their rates of reduction by 10 mM DTT. The DNA binding activity of S-cysteinylated protein is restored rapidly by DTT, whereas the cyclic sulphenamide form regains activity

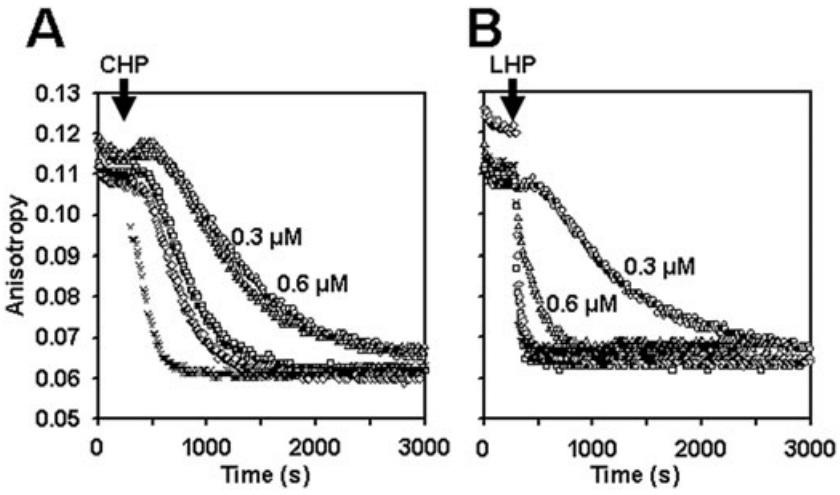

Fig. 3. Rapid inactivation of OhrR by LHP *in vitro*. The functional inactivation of OhrR (300 nM) was monitored using fluorescence anisotropy and a fluorescent probe corresponding to the *ohrA* operator site. The OhrR–DNA complex has a higher anisotropy than the free DNA probe. At the time indicated by the black arrow (300 s) protein inactivation was initiated by addition of either CHP (A) or LHP (B). These reactions were performed in the absence of added low molecular weight thiols, so functional inactivation proceeds by either reaction 4 (sulphenamide formation) or reaction 6 (overoxidation). Concentrations of oxidant were (in order of increasing inactivation rate) 0.3, 0.6, 1.5, 2 and 30 μM for CHP and 0.3, 0.6, 1.5, 2 and 3 μM for LHP. The rate of inactivation in the presence of one molar equivalent of oxidant (0.3 μM) is essentially identical for CHP and LHP, consistent with the hypothesis that inactivation proceed via slow formation of the protein sulphenamide (reaction 4). Note that the shoulder on these curves corresponds to the accumulation of protein sulphenate, as described previously (Lee *et al.*, 2007). The rates of inactivation with two molar equivalents of oxidant (0.6 μM) differ greatly, indicative of a change in mechanism for protein inactivation.

slowly. In contrast, overoxidized OhrR is irreversibly inactivated under these conditions.

We took advantage of these differences to indirectly monitor the products of OhrR oxidation in reactions containing either LHP or CHP. For example, the oxidation of OhrR by 3 μM CHP in the presence of 1 mM cysteine led to quantitative S-cysteinylation and DNA binding activity was fully and rapidly recovered by treatment with 10 mM DTT (Fig. 4A). In the absence of cysteine, the inactivation rate was much slower (primarily owing to the slow rate of cyclic sulphenamide formation). Moreover, when this sample was treated with 10 mM DTT, approximately 60% of the DNA binding activity is recovered, albeit slowly (Fig. 4A). This corresponds to the fraction of the protein present as the cyclic sulphenamide (Lee *et al.*, 2007). The remaining fraction corresponds to OhrR that is overoxidized under these conditions. These assignments have all been confirmed by ESI-MS analyses as reported previously (Lee *et al.*, 2007) and are presented here for comparison purposes.

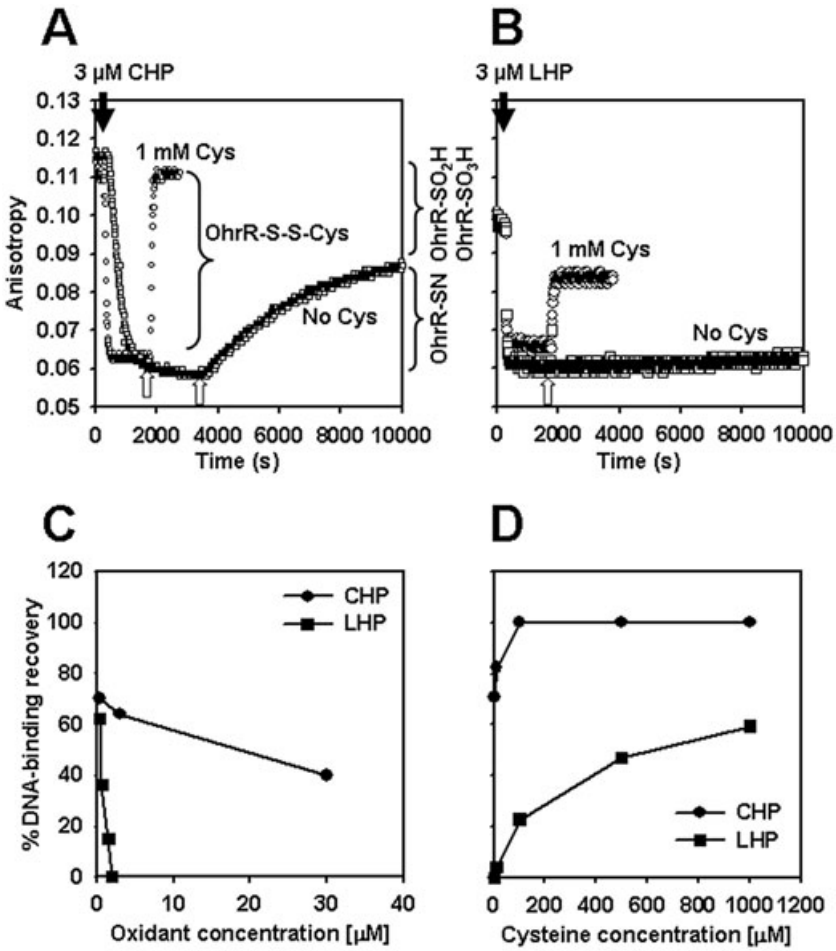

Fig. 4. Inactivation of OhrR by LHP is largely irreversible. Reversibility of OhrR inactivation was monitored using FA following oxidation (by CHP or LHP) and reduction (by 10 mM DTT).

A. Reversible oxidation of OhrR by CHP. Addition of 10 mM DTT (open arrow) leads to the rapid reactivation of OhrR (300 nM) that was inactivated by 3 μM CHP in the presence of 1 mM Cys (open circles) thereby leading to mixed-disulphide formation. In contrast, DTT only slowly and partially restores activity to protein oxidized in the absence of Cys (open squares). This is consistent with the observation that the major product under these conditions is the protein sulphenamide (S–N) and the residual irreversibly oxidized fraction corresponds to overoxidized protein (see Lee *et al.*, 2007).

B. Overoxidation of OhrR by LHP. Addition of 10 mM DTT (open arrow) leads to only partial reactivation of OhrR (300 nM) after treatment with LHP even in the presence of 1 mM Cys (open circles). In the absence of Cys (open squares), there is no recovery of protein activity, consistent with overoxidation of OhrR rather than sulphenamide formation.

C. Concentration dependence of OhrR overoxidation (in competition with sulphenamide formation). Reactivation of OhrR (300 nM) by 10 mM DTT was monitored after treatment with CHP (0.3, 3 or 30 μM) or LHP (0.3, 0.6, 1.5 or 2 μM) in the absence of Cys. Even low concentrations of LHP irreversibly inactivated OhrR.

D. Concentration dependence of OhrR overoxidation (in competition with S-cysteinylation). OhrR (300 nM) was treated with either 3 μM CHP or 3 μM LHP in the presence of the indicated concentration of Cys. The ability of 10 mM DTT to restore DNA binding activity was monitored after 30 min of oxidation. Even low levels of Cys completely protect OhrR against overoxidation by CHP, but not by LHP.

The results were significantly different when the oxidant was LHP instead of CHP (Fig. 4B). When the protein was oxidized in the absence of cysteine, the inactivation process was irreversible: there was no recovery of DNA binding activity upon incubation with 10 mM DTT (Fig. 4B), consistent with the overoxidation of OhrR observed *in vivo* under similar conditions (Fig. 2B). Strikingly, even the presence of 1 mM cysteine failed to completely trap the initially formed protein sulphenate as the reversibly S-cysteinylated product as shown by the incomplete recovery of DNA binding activity upon addition of 10 mM DTT. These observations are consistent with the hypothesis that LHP rapidly overoxidizes OhrR as noted above.

Kinetic competition between LHP-mediated overoxidation and cyclic sulphenamide formation

To monitor the kinetics of LHP and CHP overoxidation, we performed experiments equivalent to those in Fig. 4A and B at a range of oxidant and cysteine concentrations. After oxidation, the fraction of reversibly oxidized protein was measured by overnight incubation with 10 mM DTT. In the presence of only one molar equivalent of oxidant (Fig. 4C; leftmost data points), OhrR was reversibly inactivated to an approximately equivalent extent (~60–70%) by either CHP or LHP. As noted above, one molar equivalent of oxidant is only sufficient for oxidation to the sulphenate and the subsequent loss of DNA binding activity is predominantly due to the slow cyclization to the sulphenamide (as seen in Fig. 3). Using mass spectrometry, we reported previously that oxidation of OhrR by one molar equivalent of CHP leads to ~60–70% sulphenamide formation (in the absence of LMW thiols) with the remaining protein overoxidized to the sulphinic acid derivative, owing to either residual oxidant or air oxidation (Lee *et al.*, 2007). Oxidation with one equivalent of LHP also leads to the protein sulphenamide as the predominant end-product (Figs 3B and 4C).

Strikingly, with only 2 μM LHP there was no reversible oxidation and 10 mM DTT could not restore DNA binding activity (Fig. 4C). Thus, the rapid rate of overoxidation by LHP (irreversible inactivation) completely precluded formation of the cyclic sulphenamide. In contrast, even with high concentrations of CHP (30 μM) a significant fraction (~40%) of the protein was in a form that could be re-activated by prolonged incubation with DTT. Note that the fraction of the protein present as the sulphenamide (as deduced from Fig. 4C) was roughly equivalent in reactions with 30 μM CHP or 600 nM LHP (corresponding to ~300 nM LHP after consumption of 300 nM LHP to form the OhrR sulphenate). This can be expressed as: k_6(0.3 μM LHP) ~ k_6(30 μM CHP). As LHP was not in excess over protein in these reactions, the actual concentration of oxidant was decreasing significantly during the course of the reaction and a pseudofirst order treatment of the kinetic data will underestimate the true second order rate constant. Thus, these data suggest that the second order rate constant for overoxidation of the sulphenate to the sulphinic acid (k_6) is >100 times faster for LHP than for CHP.

Kinetic competition between LHP-mediated overoxidation and S-cysteinylation

The relatively stable OhrR sulphenate can be trapped by S-cysteinylation with a second order rate constant k_2 of ~10^3 M^{-1} s^{-1} (Lee *et al.*, 2007). As a result, even low levels of cysteine suffice to protect OhrR from overoxidation in the presence of CHP (Fig. 4D and Lee *et al.*, 2007). In contrast, treatment of OhrR with 3 μM LHP in the presence of even 1 mM cysteine led to a partial (~60%) conversion of OhrR to the reversibly inactivated, S-cysteinylated derivative (Fig. 4B versus Fig. 4A).

To more carefully quantify the kinetic competition between S-cysteinylation and overoxidation, we monitored the partitioning between reversibly and irreversibly oxidized OhrR after treatment with 3 μM oxidant (either CHP or LHP) in the presence of various cysteine concentrations (Fig. 4D). At a concentration of 100 μM cysteine, OhrR treated with 3 μM CHP was quantitatively S-cysteinylated and activity could be fully restored by DTT. In contrast, only ~20% of OhrR treated with 3 μM LHP under these conditions was reversibly oxidized. Indeed, with 3 μM LHP, full recovery of DNA binding could not be obtained even in the presence of 5 mM cysteine (data not shown). Assuming that the rate constant for S-cysteinylation is unaffected by the nature of the oxidant, these results are consistent with the observation that the competing reaction (overoxidation) is much faster for LHP as compared with CHP. This is in agreement with the kinetic competition studies above in which overoxidation rates (k_6) were compared with sulphenamide formation (k_4).

Kinetic description of OhrR oxidation and inactivation by organic peroxides

Our current estimates for the rate constants involved in the oxidative inactivation of OhrR by LHP and CHP are summarized in Table 1. The most accurately measured rates are those for sulphenamide formation (k_4), the second order rate constant for S-cysteinylation (k_2), and the overall rate of functional inactivation by CHP in the presence of excess cysteine (k_1) (Lee *et al.*, 2007). As both k_2 and k_4 are known, and the effective (pseudofirst order) rate of the former reaction can be controlled by cysteine concentration, we can provide a reasonably accurate estimate of k_6, the second order rate constant for overoxidation of OhrR by LHP. For example, as the rate of reaction of the

Table 1. Estimated rates (and half-times) for reaction of OhrR with CHP and LHP.

Reaction	Rate	CHP	LHP	$t_{1/2}$ (CHP)	$t_{1/2}$ (LHP)
Oxidation	k_1	$> 2 \times 10^4$ M^{-1} s^{-1}	$> 10^6$ M^{-1} s^{-1}	0.3 s (100 μM CHP)	< 0.1 s (5 μM LHP)
Overoxidation	k_6	~200 M^{-1} s^{-1}	2.6×10^5 M^{-1} s^{-1}	35 s (100 μM CHP)	~0.5 s (5 μM LHP)
S-cysteinylation	k_2	1×10^3 M^{-1} s^{-1}		3.5 s (200 μM cys)	
Sulphenamide formation	k_4	1.3×10^{-3} s^{-1}		9 min	

initial OhrR sulphenate with 3 μM LHP is approximately the same as that for 800 μM cysteine (~50% reversible oxidation; Fig. 4D), we estimate k_6 as ~2.6×10^5 M^{-1} s^{-1} for LHP (and much slower with CHP). As k_6 is less than k_1 (see above), this also provides a lower limit for the value of k_1 for LHP. We previously estimated the initial rate of oxidation of OhrR by CHP as at least 2×10^4 M^{-1} s^{-1} based on the rate of functional inactivation in the presence of saturating levels of cysteine. As shown here, the rate of initial oxidation is at least 10-fold faster with LHP.

Using these estimated rate constants, we can calculate half-times for each of the reactions involved in the inactivation of OhrR in cells exposed to CHP and LHP (Table 1). Both oxidants, when added at their most effective concentrations, are estimated to oxidize OhrR in less than 1 s (assuming rapid penetration of oxidant into the cell). Functional inactivation of OhrR by S-cysteinylation is also predicted to occur rapidly (on a time scale of seconds). The actual rates may differ *in vivo* as there are additional LMW thiols in the cell, and the concentrations of all relevant thiol species are not accurately known. Here we have used 200 μM as a reasonable estimate of the intracellular concentration of LMW thiols as detected using monobromobimane labelling (e.g. (Newton *et al.*, 1996). In LHP-treated cells, the rate of overoxidation is estimated to be faster than S-cysteinylation consistent with the observed *in vivo* product distribution (Fig. 2B). Note that the rate of sulphenamide formation measured *in vitro* is too slow to effectively compete with S-cysteinylation, although small amounts of protein sulphenamide are detected *in vivo* (Lee *et al.*, 2007). The observation that OhrR accumulates *in vivo* as the sulphinic acid derivative in cells treated with LHP (Fig. 2B), whereas it accumulates predominantly as S-thiolated derivatives in cells treated with CHP (Fig. 2A and Lee *et al.*, 2007), indicates that these kinetic inferences have relevance for the *in vivo* pathways of OhrR inactivation. We conclude that OhrR, like the hydrogen peroxide sensors OxyR and PerR (Aslund *et al.*, 1999; Lee and Helmann, 2006), is an extremely sensitive regulatory protein with a second order rate constant for inactivation by LHP of $> 10^5$ M^{-1} s^{-1}.

Concluding remarks

OhrR is exquisitely sensitive to oxidation by low levels of organic peroxides and effectively insensitive to H_2O_2. Indeed, it was previously shown that inactivation of OhrR by H_2O_2 required high concentrations of oxidant (at least 100 μM H_2O_2) and could only be observed in cells lacking the two major H_2O_2 detoxification enzymes: catalase and alkyl hydroperoxide reductase (Fuangthong and Helmann, 2002). Here, we demonstrate that full induction of the OhrR-regulated *ohrA* gene is achieved with either 100 μM CHP or 5 μM LHP.

Analysis of OhrR inactivation by CHP and LHP suggests that this regulator may switch between reversible and irreversible inactivation pathways depending on the nature of the oxidant. The *in vivo* product of LHP oxidation is predominantly the sulphinic (and likely sulphonic) acid derivative of OhrR (Fig. 2B), which represents an irreversible oxidation event in bacteria. Although sulphinic acids can be repaired in at least some proteins in eukaryotes by sulphiredoxin (Biteau *et al.*, 2003), an equivalent protein repair system has not been detected in bacteria. In contrast, oxidation of OhrR by CHP leads to S-thiolated species as the primary reaction product (Fig. 2B and Lee *et al.*, 2007). These can be repaired by thiol–disulphide exchange reactions. Indeed, the S-thiolated species detected in cells after 2 min of treatment with CHP decrease over time and are virtually undetectable by 15 min post treatment, consistent with rapid *in vivo* repair (data not shown). Thus, our *in vivo* and *in vitro* results suggest that, depending on the nature of the oxidant, OhrR can function as either a reversible or an irreversible (sacrificial) regulator. Sacrificial regulators are fairly rare, but well-characterized examples in *Escherichia coli* include the cleavable LexA repressor and the modification of the Ada repressor. Furthermore, the *B. subtilis* PerR protein is inactivated by protein oxidation events that are thought to be irreversible (Lee and Helmann, 2006). It is interesting to note that all of these proteins regulate stress responses that may determine the survival of the cell, and therefore rapid induction may be more important in this situation than the energetic cost of destroying an inactivated repressor.

Experimental procedures

Monitoring OhrR oxidation in B. subtilis

OhrR-FLAG was generated as previously described (Lee *et al.*, 2007). A total of 200 ml of cells in LB at OD_{600} ~0.4 was untreated or treated with 100 μM CHP or 5 μM LHP for 1 min.

OhrR-FLAG was recovered in the presence of 100 mM IA, isolated by SDS-PAGE and then digested with trypsin. The resulting tryptic peptides were analysed using an Applied Biosystems 4700 MALDI-TOF mass spectrometer as described previously (Lee *et al*., 2007).

Transcriptional fusion analysis

Cells were grown in LB at 37°C with aeration until OD_{600} ~0.4. The cells were treated with the indicated concentration of CHP or LHP for 15 min. Samples were harvested and assayed for β-galactosidase activities as previously described (Miller, 1972).

Synthesis of LHP

LHP was generated *in vitro* as described (Evans *et al*., 1998). The mixture of 0.3 mM linoleic acid (Sigma) and soybean lipoxygenase (4000 U; Sigma) in 0.1 M sodium borate buffer (pH 9) was stirred vigorously for 30 min at room temperature. The product was loaded onto an end-capped C18 reverse-phase column (Strata), and LHP was eluted in 5 ml of methanol. The functional concentration of LHP was calculated based on inactivation of OhrR in an FA assay using CHP as standard.

Fluorescence anisotropy

A 6-carboxyfluorescein-(6F-) labelled DNA fragment containing the *ohrA* operator site was generated by annealing 5′-6F-TACAATTAAATTGTATACAATTAAATTGTA-3′ (Integrated DNA Technologies) and its unlabelled complementary strand. FA measurements (λ_{ex} = 495 nm; slit width = 15 nm, λ_{em} = 520 nm; slit width = 20 nm, integration time = 1 s) were performed with 50 nM DNA and 300 nM OhrR (monomer) in 3 ml of 20 mM Tris (pH 8.0) containing 150 mM NaCl, and 5% (v/v) glycerol unless otherwise noted. FA values were recorded automatically every 10 s using a Perkin-Elmer LS55 luminescence spectrometer. The g-factor for the experiments was 1.07 ± 0.01.

Acknowledgements

This work was supported by a grant from the National Science Foundation (MCB-0640616).

References

Aslund, F., Zheng, M., Beckwith, J., and Storz, G. (1999) Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol-disulfide status. *Proc Natl Acad Sci USA* **96:** 6161–6165.

Biteau, B., Labarre, J., and Toledano, M.B. (2003) ATP-dependent reduction of cysteine-sulphinic acid by *S. cerevisiae* sulphiredoxin. *Nature* **425:** 980–984.

Chen, L., Keramati, L., and Helmann, J.D. (1995) Coordinate regulation of *Bacillus subtilis* peroxide stress genes by hydrogen peroxide and metal ions. *Proc Natl Acad Sci USA* **92:** 8190–8194.

Evans, M.V., Turton, H.E., Grant, C.M., and Dawes, I.W. (1998) Toxicity of linoleic acid hydroperoxide to *Saccharomyces cerevisiae*: involvement of a respiration-related process for maximal sensitivity and adaptive response. *J Bacteriol* **180:** 483–490.

Fang, F.C. (2004) Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat Rev Microbiol* **2:** 820–832.

Fuangthong, M., and Helmann, J.D. (2002) The OhrR repressor senses organic hydroperoxides by reversible formation of a cysteine-sulfenic acid derivative. *Proc Natl Acad Sci USA* **99:** 6690–6695.

Fuangthong, M., Atichartpongkul, S., Mongkolsuk, S., and Helmann, J.D. (2001) OhrR is a repressor of *ohrA*, a key organic hydroperoxide resistance determinant in *Bacillus subtilis*. *J Bacteriol* **183:** 4134–4141.

Fuangthong, M., Herbig, A.F., Bsat, N., and Helmann, J.D. (2002) Regulation of the *Bacillus subtilis* fur and perR genes by PerR: not all members of the PerR regulon are peroxide inducible. *J Bacteriol* **184:** 3276–3286.

Gaballa, A., and Helmann, J.D. (2002) A peroxide-induced zinc uptake system plays an important role in protection against oxidative stress in *Bacillus subtilis*. *Mol Microbiol* **45:** 997–1005.

Gusarov, I., and Nudler, E. (2005) NO-mediated cytoprotection: instant adaptation to oxidative stress in bacteria. *Proc Natl Acad Sci USA* **102:** 13855–13860.

Helmann, J.D., Wu, M.F., Gaballa, A., Kobel, P.A., Morshedi, M.M., Fawcett, P., and Paddon, C. (2003) The global transcriptional response of *Bacillus subtilis* to peroxide stress is coordinated by three transcription factors. *J Bacteriol* **185:** 243–253.

Herbig, A.F., and Helmann, J.D. (2001) Roles of metal ions and hydrogen peroxide in modulating the interaction of the *Bacillus subtilis* PerR peroxide regulon repressor with operator DNA. *Mol Microbiol* **41:** 849–859.

Imlay, J.A. (2003) Pathways of oxidative damage. *Annu Rev Microbiol* **57:** 395–418.

Jacob, C., Knight, I., and Winyard, P.G. (2006) Aspects of the biological redox chemistry of cysteine: from simple redox responses to sophisticated signalling pathways. *Biol Chem* **387:** 1385–1397.

Kiley, P.J., and Storz, G. (2004) Exploiting thiol modifications. *PLoS Biol* **2:** e400.

Klomsiri, C., Panmanee, W., Dharmsthiti, S., Vattanaviboon, P., and Mongkolsuk, S. (2005) Novel roles of ohrR-ohr in Xanthomonas sensing, metabolism, and physiological adaptive response to lipid hydroperoxide. *J Bacteriol* **187:** 3277–3281.

Lee, J.W., and Helmann, J.D. (2006) The PerR transcription factor senses H2O2 by metal-catalysed histidine oxidation. *Nature* **440:** 363–367.

Lee, J.W., and Helmann, J.D. (2007) Functional specialization within the Fur family of metalloregulators. *Biometals* **20:** 485–499.

Lee, J.W., Soonsanga, S., and Helmann, J.D. (2007) A complex thiolate switch regulates the *Bacillus subtilis* organic peroxide sensor OhrR. *Proc Natl Acad Sci USA* **104:** 8743–8748.

Miller, J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Mongkolsuk, S., and Helmann, J.D. (2002) Regulation of inducible peroxide stress responses. *Mol Microbiol* **45:** 9–15.

van Montfort, R.L., Congreve, M., Tisi, D., Carr, R., and Jhoti, H. (2003) Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B. *Nature* **423:** 773–777.

Newberry, K.J., Fuangthong, M., Panmanee, W., Mongkolsuk, S., and Brennan, R.G. (2007) Structural mechanism of organic hydroperoxide induction of the transcription regulator OhrR. *Mol Cell* **28:** 652–664.

Newton, G.L., Arnold, K., Price, M.S., Sherrill, C., Delcardayre, S.B., Aharonowitz, Y., *et al.* (1996) Distribution of thiols in microorganisms: mycothiol is a major thiol in most actinomycetes. *J Bacteriol* **178:** 1990–1995.

Panmanee, W., Vattanaviboon, P., Eiamphungporn, W., Whangsuk, W., Sallabhan, R., and Mongkolsuk, S. (2002) OhrR, a transcription repressor that senses and responds to changes in organic peroxide levels in *Xanthomonas campestris* pv. phaseoli. *Mol Microbiol* **45:** 1647–1654.

Panmanee, W., Vattanaviboon, P., Poole, L.B., and Mongkolsuk, S. (2006) Novel organic hydroperoxide-sensing and responding mechanisms for OhrR, a major bacterial sensor and regulator of organic hydroperoxide stress. *J Bacteriol* **188:** 1389–1395.

Salmeen, A., Andersen, J.N., Myers, M.P., Meng, T.C., Hinks, J.A., Tonks, N.K., and Barford, D. (2003) Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate. *Nature* **423:** 769–773.

Torres, M.A., and Dangl, J.L. (2005) Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. *Curr Opin Plant Biol* **8:** 397–403.

Yang, J., Groen, A., Lemeer, S., Jans, A., Slijper, M., Roe, S.M., *et al.* (2007) Reversible oxidation of the membrane distal domain of receptor PTPalpha is mediated by a cyclic sulfenamide. *Biochemistry* **46:** 709–719.

