Transcriptional Regulator of Oxidative Stress-Inducible Genes: Direct Activation by Oxidation

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The *oxyR* gene positively regulates genes induced by oxidative stress in *Salmonella typhimurium* and *Escherichia coli*. Purification of the OxyR protein showed that oxidized but not reduced OxyR activates transcription of oxidative stress-inducible genes in vitro. Conversion between the two forms of OxyR is rapid and reversible. Both the oxidized and the reduced forms of the OxyR protein are capable of binding to three diverse sequences upstream of OxyR-regulated promoters, but the interactions of the two forms of OxyR with the promoter regions are different. The results suggest that direct oxidation of the OxyR protein brings about a conformational change by which OxyR transduces an oxidative stress signal to RNA polymerase.

THE MECHANISMS BY WHICH CELLS SENSE ENVIRONMENTAL adversity and then transduce the stress signals into a change in gene expression are known for only a limited number of responses. MerR, a regulator of mercury resistance in *Escherichia coli*, is activated to induce mercuric reductase upon binding mercury (*1*). The *E. coli* Ada protein, which regulates the expression of genes in response to DNA methylation, is activated by the transfer of a methyl group from the DNA to the Ada protein (*2*). For other environmental stresses, the transcriptional regulator has been characterized, but little is known about how the environmental signal is transmitted to the transcriptional regulator. For example, the level of RpoH, a regulator of the heat shock response in *E. coli*, is increased after a shift to higher temperature (*3*), and the transcriptional activity of the heat shock factor in *Saccharomyces cerevisiae* is thought to be modulated by phosphorylation (*4*), but the actual sensors of heat shock are still unknown.

The cellular response to oxidative stress is of importance since reactive oxygen species including superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxy radical ($HO\cdot$), have been implicated as causative agents in several degenerative diseases (*5*). Reactive oxygen species can be produced by the incomplete reduction of oxygen during respiration, by exposure to radiation or to oxidation-reduction (redox) active drugs such as paraquat, or by release from macrophages in response to bacterial invasion (*5*). They can lead to damage of almost all cell components—DNA, lipid membranes, and proteins (*5*). Both prokaryotic and eukaryotic cells have inducible defenses to counter oxidative damage (*6–8*), but the mechanisms by which cells receive and respond to oxidative stress have not yet been elucidated.

The *oxyR*-controlled regulon of hydrogen peroxide–inducible genes in *Salmonella typhimurium* and *E. coli* has provided a model for studying the cellular response to oxidative stress. When bacterial cells are treated with low doses of hydrogen peroxide, the synthesis of at least 30 proteins is induced, and the cells become resistant to subsequent doses of hydrogen peroxide that would otherwise be lethal (*7–9*). The expression of nine of the proteins induced by hydrogen peroxide treatment is under the control of the *oxyR* gene (*8*). Strains carrying deletions of *oxyR* are unable to induce the nine proteins and are hypersensitive to hydrogen peroxide and other oxidants (*8*). Several of the proteins whose expression is regulated by *oxyR* have been identified and include catalase, encoded by *katG*, and an alkyl hydroperoxide reductase, encoded by *ahpC* and *ahpF* (*8, 9*).

Sequence analysis of the *oxyR* gene revealed that OxyR is a member of a large family of bacterial regulators that includes the *E. coli* regulatory protein LysR and the *Rhizobium* regulatory protein NodD (*10–12*). Like many other members of the LysR family, OxyR acts as both a positive and a negative regulator. OxyR is an activator of *katG* and *ahpCF* expression and negatively regulates its own expression (*10, 13*). The levels of the *katG* and *ahpCF* mRNA's are greatly increased in *oxyR* mutant strains that have constitutively high levels of the *oxyR*-regulated gene products suggesting that OxyR regulates at the level of transcription (*9, 13*). Extracts of strains that overproduce the OxyR protein protect regions upstream of the *oxyR*-regulated *oxyR*, *katG*, and *ahpC* promoters from deoxyribonuclease (DNase) I digestion although the protected sequences show very little sequence similarity (*13*). Bölker and Kahmann have also found that OxyR is a repressor of the *mom* gene (encoding a DNA modification function) of phage Mu (*11*). To elucidate the mechanisms by which bacterial cells sense oxidative stress and then induce a defense response, we purified the OxyR protein to homogeneity and studied its ability to bind *oxyR*-regulated promoters and to activate transcription in vitro.

No increase in amount or synthesis of OxyR after treatment with hydrogen peroxide. The expression of *oxyR*-regulated proteins as observed on two-dimensional gels, is induced within 10 minutes after treatment with hydrogen peroxide (*8*). The expression

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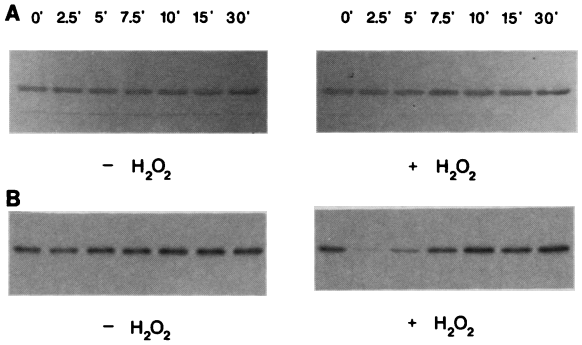

Fig. 1. Effect of hydrogen peroxide on the amount and synthesis of OxyR. (**A**) Cultures of TA4471 growing in Vogel-Bonner medium (*29*) containing 0.4 percent glucose and 0.01 percent (each) arginine, aspartate, glutamate, and methionine, were treated with hydrogen peroxide (*8, 9*). At the indicated times, 200-μl samples were centrifuged, resuspended in Laemmli buffer (*30*), and subjected to electrophoresis on 12 percent polyacrylamide gels. The proteins were transferred to nitrocellulose filters by electroblotting, and the filter was probed with a 1:2000 dilution of antibodies to an OxyR–β-galactosidase fusion protein. Bound antibody was visualized by the reaction catalyzed by alkaline phosphatase conjugated with goat antiserum to rabbit antibodies (*31*). A 1.2-kb Ssp I–Eco RV fragment of pAQ17 (*10*) carrying *oxyR* was cloned into the Sma I site of pEX2 (*32*) to generate the OxyR–β-galactosidase fusion protein of approximately 150 kD. The fusion protein was separated from other *E. coli* proteins in a cell extract on a 6 percent polyacrylamide gel. The portion of the gel containing the fusion protein was excised, and the protein was eluted from the gel fragment with an Elutrap apparatus (Schleicher & Schuell). The fusion protein (200 μg) was mixed with Freund's complete adjuvant, sonicated to form an emulsion, and injected into a New Zealand white female rabbit. The rabbit was reinjected twice (at 15-day intervals) with additional fusion protein (200 μg) mixed with Freund's incomplete adjuvant. Serum obtained 15 days after the third injection reacted predominantly with the 34-kD protein not detected in *oxyR* deletion strains. Control immunoblots showed that less than twofold differences in the amounts of OxyR could be detected. (**B**) Cells (1 ml) with and without treatment with hydrogen peroxide were labeled with 35 μCi of [^{3}H]leucine for 2-minute intervals (−2 to 0, 0.5 to 2.5, 3 to 5, 5.5 to 7.5, 8 to 10, 13 to 15, and 28 to 30). The labeling was stopped by the addition of 25 μl of 1 percent leucine, and the reactions were placed on ice. Cells were then lysed, and OxyR was precipitated with 5 μl of anti-OxyR (1:200 dilution) as described (*33*).

of an *oxyR*-regulated *katG-lacZ* fusion is also elevated within 5 minutes after treatment with hydrogen peroxide (*13*). To determine whether the increased expression of *oxyR*-regulated genes was associated with an increase in OxyR, we treated exponentially growing cells with hydrogen peroxide and examined the amount of OxyR and rate of OxyR synthesis. We first examined the amount of OxyR in an *oxyR* deletion strain carrying *oxyR* on a multicopy plasmid (TA4471, *oxyRΔ3*/pAQ17) (*14*). On this plasmid, *oxyR* is under the control of its own promoter, and the *oxyR* deletion strain carrying the plasmid shows the same adaptation to hydrogen peroxide as the corresponding wild-type strain (*10*). The TA4471 strain was grown in minimal medium and half of the culture was treated with 60 μ*M* hydrogen peroxide. Samples of the treated and untreated cells were taken immediately before the treatment and at intervals after the treatment, and were then subjected to SDS-gel electrophoresis, transferred to nitrocellulose, and probed with antibodies to an OxyR–β-galactosidase fusion protein (Fig. 1A). These immunoblots showed that there is no significant increase in the amount of OxyR after the treatment with hydrogen peroxide. Similarly, the levels of OxyR in a wild-type *E. coli* K12 strain carrying a single copy of the *oxyR* gene remained constant after treatment with hydrogen peroxide (*15*).

A culture of TA4471, with and without treatment with hydrogen peroxide, was also labeled with [^{3}H]leucine for 2-minute intervals before and after treatment with hydrogen peroxide. Portions of the cells were lysed, and the OxyR protein was precipitated with the antibody to OxyR (anti-OxyR) and subjected to electrophoresis (Fig. 1B). Whereas the synthesis of OxyR was unchanged in untreated cells, cells treated with hydrogen peroxide showed a decrease rather than an increase in OxyR synthesis during the first 10 minutes after treatment with hydrogen peroxide.

These results suggest that increased expression of the *oxyR* regulon after treatment with hydrogen peroxide is not a result of increased levels or synthesis of OxyR, but is rather a consequence of a modification of preexisting OxyR. This conclusion is in agreement with other observations about the *oxyR*-mediated response. Strains (D1210/pAQ25) (Fig. 2A) that overproduce the OxyR protein, do

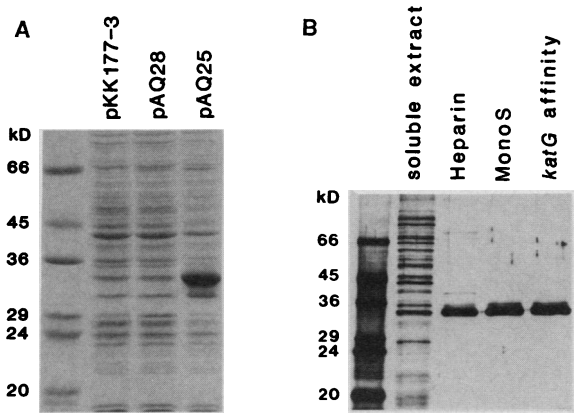

Fig. 2. Overproduction and purification of OxyR. (**A**) Samples (60 μl) of overnight cultures of D1210 (HB101/F′*lacI*^{q}) carrying pKK177-3 (*tac* promoter) (*34*), pAQ28 (*tac* promoter–*oxyR*), and pAQ25 (*tac* promoter–efficient Shine-Dalgarno–*oxyR*) were centrifuged, resuspended in Laemmli buffer, and subjected to electrophoresis on a 12 percent polyacrylamide gel. The 1.5-kb Eco RI–Hind III fragment of pAQ17 (*10*) carrying *oxyR* was cloned into the corresponding sites of pKK177-3 to generate pAQ28. To generate pAQ25, the 1.5-kb Eco RI–Eco RV fragment of pAQ17 was cloned into the Eco RI–Hinc II sites of a pUC18 derivative in which the sole Ssp I site was removed by limited Bal 31 digestion. The Eco RI–Ssp I fragment carrying the *oxyR* promoter was then replaced by annealed oligonucleotides (5′-ATTCATATTATTTCTCCTTTG-3′ and 5′-AATTC-AAAGGAGAAATAATATGAAT-3′) without alteration of the *oxyR* coding sequence. Finally the 1.5-kb Eco RI–Hind III fragment carrying *oxyR* with the altered Shine-Dalgarno sequence (*16*) was subcloned into pKK177-3. (**B**) Samples [0.1 μg of protein as determined by Bradford assays (*35*) with bovine serum albumin (BSA) as a standard] of the soluble starting material, peak heparin agarose, MonoS, and *katG* affinity column fractions were subjected to SDS electrophoresis and silver-stained (*36*). Whereas a few contaminating protein bands were barely visible for the MonoS fraction, no bands other than OxyR were seen for the *katG* affinity fraction. The fractions represented were treated as follows: 5 g of cells, obtained from 10 liters of D1210/pAQ25, treated with 250 mg of isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 hours, were resuspended in 20 ml of 50 m*M* Hepes, *p*H 7.6, 0.1 m*M* EDTA, 0.5 m*M* dithiothreitol (DTT), 2 m*M* $MgCl_2$, and 50 m*M* KCl. The cells were lysed by three passages through a French pressure cell. Chromosomal DNA was then digested with DNase I, and the insoluble fraction was removed by centrifugation. The buffer concentrations of the soluble fraction were adjusted to 50 m*M* Hepes, *p*H 7.6, 0.5 m*M* EDTA, 1 m*M* DTT, 10 m*M* $MgCl_2$ (buffer Z), and 0.1*M* KCl and applied to 150 ml of heparin Sepharose CL-6B (Pharmacia) at 1 to 3 ml/min. The column was washed with 500 ml of buffer Z containing 0.1*M* KCl and 500 ml of buffer Z containing 0.2*M* KCl and then eluted with a 700-ml linear gradient of buffer Z containing 0.2 to 0.5*M* KCl. The 10-ml fraction that contained the peak of OxyR and eluted at 0.35 to 0.40*M* KCl was diluted with 40 ml of buffer Z and applied to 1 ml of Mono S HR5/5 (Pharmacia) at 0.5 ml/min. The protein was eluted with a 20-ml linear gradient of buffer Z containing KCl (0.1 to 1.0*M*). The 1-ml fraction containing the peak of OxyR was diluted with 4 ml of buffer Z containing 0.1 percent NP-40 and applied to a DNA affinity column (0.5-ml volume) prepared with oligonucleotides based on the *katG* promoter sequence (*37*) as outlined (*38*). The flowthrough was applied to the column a second time. The column was then washed with 4 ml of buffer Z containing 0.1*M* KCl and 0.1 percent NP-40, and OxyR was eluted with 2 ml of buffer Z containing 0.4*M* KCl and 0.1 percent NP-40.

not show increased expression of the *oxyR*-regulated proteins (*15*). In addition, the constitutive overproduction of *oxyR*-regulated proteins in strains carrying the *oxyR2* mutation is due to a missense mutation (Ala to Val) in OxyR rather than increased expression of the *oxyR* mRNA (*10*) or protein (*15*).

Binding of three non-homologous sequences by purified OxyR. To further characterize OxyR activation after oxidative stress, we purified the OxyR protein to homogeneity. We previously observed that OxyR negatively regulates its own expression (*10*) and found that cloning the *oxyR* promoter and open reading frame behind the well-transcribed *tac* promoter did not result in large overproduction of OxyR (Fig. 2A, pAQ28). To eliminate the site of negative autoregulation and to allow for optimal expression of the *oxyR* message, the *oxyR* promoter region (including the OxyR binding site and *oxyR* Shine-Dalgarno sequence) was replaced with an efficiently translated Shine-Dalgarno sequence (*16*) (Fig. 2A). OxyR is the predominant protein in strains carrying this construction (Fig. 2A).

Whereas a significant portion of the overproduced OxyR protein was sequestered in the insoluble fraction of the OxyR-overproducing cells, OxyR was still abundant in the soluble fraction. To avoid any complications caused by possible structural differences between the soluble and the insoluble protein, we chose to purify OxyR from only the soluble fraction. Full purification (no other bands were visible on a silver-stained gel loaded with 0.5 μg of protein) was achieved by fractionation on three columns (Fig. 2B). Fractionation on a heparin-agarose column eliminated most of the cellular proteins. A second fractionation step on MonoS resin removed additional contaminants and resulted in concentration of the OxyR protein. The remaining contaminants were eliminated on a sequence-specific DNA affinity matrix consisting of ligated oligonucleotides containing the OxyR-binding site upstream of *katG* (portions of the *katG* affinity fraction shown in Fig. 2B were used in all subsequent experiments).

Crude extracts from OxyR-overproducing cells protect sequences upstream of the *katG, ahpC*, and *oxyR* genes from DNase I digestion that do not show any apparent sequence similarity (*17*). To determine whether OxyR purified to homogeneity on the basis of binding to the *katG* sequence could still bind the *ahpC* and *oxyR* sequences, we incubated samples of the fraction shown in Fig. 2B with *katG, ahpC*, and *oxyR* promoter fragments and assayed for the ability to protect against DNase I digestion. Samples containing less than 5 ng of purified OxyR were able to fully protect all of the promoter fragments against DNase I digestion (*18*) (Fig. 5). These results showed that purified OxyR is capable of binding to the three different sequences at the *katG, ahpC*, and *oxyR* promoters. The sites bound by OxyR are quite large (>45 nucleotides) suggesting that OxyR might bind as a multimer, but no obvious inverted or tandem repeats are seen within the protected regions.

OxyR activates transcription on oxidation. To investigate the

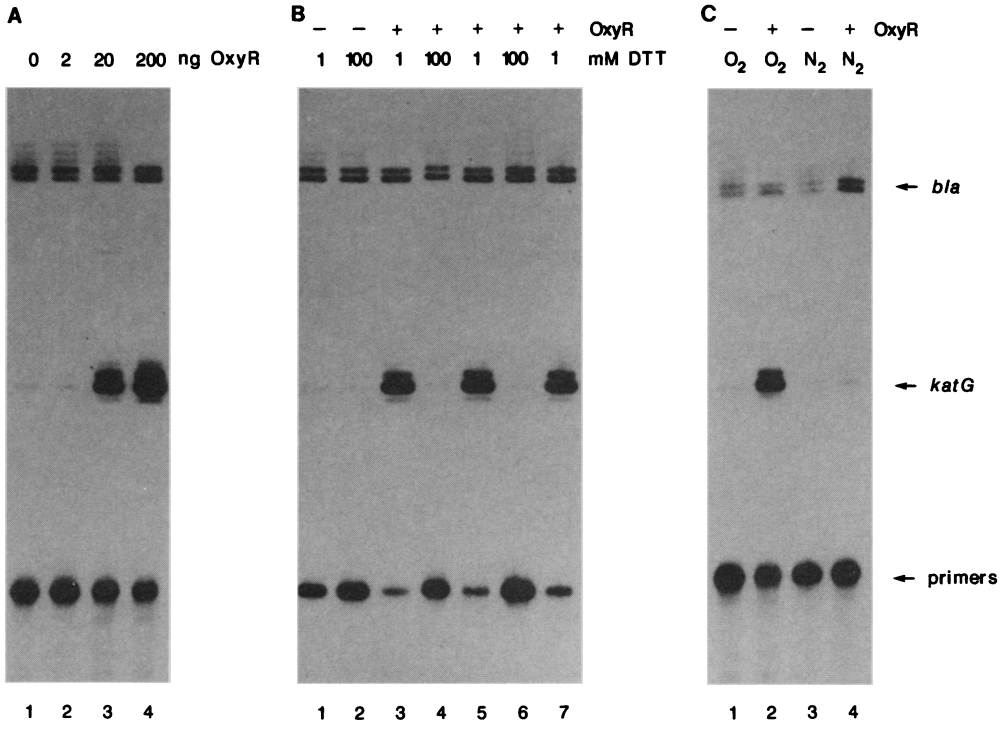

Fig. 3. Activation of *katG* expression in vitro by oxidized but not reduced OxyR. (**A**) Increasing amounts [2, 20, and 200 ng of protein based on Bradford assays (*35*) before the centrifugation step below] of purified OxyR were added to RNA polymerase and a plasmid (pBT22) (*19*) carrying the *katG* and *bla* genes, and the resulting in vitro transcription products were examined by primer extension. The transcription assays were carried out as follows: A sample (50 μl) of purified OxyR with carrier BSA (15 μg) was centrifuged through 800 μl of Sephadex (Boehringer Mannheim) into the transcription buffer (40 m*M* tris-Cl, *p*H 7.9, 0.1*M* KCl, 10 m*M* $MgCl_2$) containing 1 m*M* DTT, 5 percent glycerol, and 0.1 percent NP-40. A portion (5 μl) of centrifuged OxyR together with 2.5 μl of H_2O was then incubated with pBT22 DNA (0.2 μg in 36.5 μl of transcription buffer) for 10 minutes at 37°C. RNA polymerase holoenzyme [0.5 μg (*39*) in 5 μl of transcription buffer] was added to the previously bound template, and the reaction was incubated for an additional 10 minutes at 37°C. After the addition of 1 μl of a 25 m*M* NTP mixture, the reactions were incubated for 5 minutes at 37°C. The reactions were terminated by the addition of phenol and extracted several times with phenol and chloroform. Then a labeled primer was added and extended with reverse transcriptase (*10, 13*). (**B**) OxyR was assayed for the ability to activate *katG* transcription in the presence of 1 or 100 m*M* DTT. The OxyR fractions represented in lanes 3 through 7 were treated as follows. Purified OxyR (50 μl) with BSA was centrifuged into transcription buffer containing 1 m*M* DTT, glycerol, and NP-40 as described above. After a sample was removed (lane 3), the DTT concentration was adjusted to 100 m*M*. Another sample was removed (lane 4) before the remainder was centrifuged through a second Sephadex column equilibrated with transcription buffer containing 1 m*M* DTT, glycerol, and NP-40. After a third sample (lane 5) was removed OxyR was again treated with 100 m*M* DTT (lane 6) and subsequently centrifuged into 1 m*M* DTT (lane 7). The OxyR samples [1 μl (lane 3), 1.5 μl (lane 4), 3 μl (lane 5), 3.5 μl (lane 6), and 5 μl (lane 7), to compensate for dilution by the added DTT or for loss during centrifugation] were then assayed for transcription as described for (A). The DTT concentrations of the transcription reactions corresponding to lanes 2, 4, and 6 were adjusted to 100 m*M* by the addition of 2.5 μl of 1.7*M* DTT instead of 2.5 μl of H_2O. (**C**) OxyR (1 μl of the centrifuged sample described for (A) was assayed for the ability to activate *katG* transcription in the presence of air- or nitrogen-saturated buffers. The transcription assays for lanes 1 and 2 were performed as described for (A) except that the DTT concentration of the reaction mixture was adjusted to 10 m*M* by the addition of 2.5 μl of 0.2*M* DTT instead of 2.5 μl of H_2O. The transcription assays for lanes 3 and 4 were performed (as described for lanes 1 and 2) in the Anaerobic Facility (containing less than 5 ppm of O_2) in the Laboratory of Biochemistry at the National Heart Lung and Blood Institute.

mechanism by which OxyR regulates the expression of the *oxyR* regulon, we first assayed the ability of extracts from OxyR-overproducing cells to activate expression of the *ahp* genes in an in vitro transcription-translation assay. The addition of extracts prepared by sonication stimulated the expression of the *ahp* genes, but not a control *bla* gene (encoding β-lactamase) (*18*). Initially this finding was surprising because neither the cells nor the extracts had been treated with hydrogen peroxide. Suspecting that OxyR could be activated solely on release from the reducing environment of the cell into air-saturated buffers [even buffers containing 1 m*M* dithiothreitol (DTT)], we prepared extracts in the presence of various antioxidants and reductants. Extracts from the OxyR-overproducing strain prepared and assayed in the presence of 100 m*M* DTT no longer activated the *ahp* genes in the transcription-translation assay (*18*). The high concentration of DTT did not alter the basal levels of *ahp* or *bla* expression, and the inactive extracts could activate expression if the 100 m*M* DTT was removed by dialysis (*18*). Concentrations of DTT as low as 10 m*M* prevented the activation if extracts were prepared and assayed under semianaerobic conditions (tubes purged with argon) (*18*). These results suggested that the oxidation state of the OxyR-enriched extracts affected their ability to stimulate expression of the *ahp* genes in vitro.

To determine whether OxyR was the sole sensor of oxidation and the only component required to activate *oxyR*-regulated gene expression, we examined the ability of purified OxyR to regulate expression of *oxyR*-regulated genes in more defined transcription assays. Samples of the most highly purified OxyR fraction were incubated with RNA polymerase holoenzyme, and plasmids carrying the *oxyR*-regulated *ahpC* (pAQ27) (*13*) or *katG* genes (pBT22) (*19*) as well as the *bla* gene as a control. The transcription products generated in vitro were then examined by primer extension assays. Increasing amounts of OxyR increased the transcription of both the *ahpC* (*15*) and *katG* genes (Fig. 3A) while having no effect on the expression of the *bla* gene. Densitometer scans of Fig. 3A showed that the addition of OxyR caused an induction of the *katG* message of more than 100-fold. The start sites of the transcripts generated in vitro, as determined by the lengths of the primer extension products, agree with the transcription starts seen for the *ahpC* and *katG* genes in vivo (*13*) and described for the *bla* gene (*20*).

We also assayed the ability of purified OxyR to activate transcription in the presence of 1 and 100 m*M* DTT to determine whether the purified protein showed the same sensitivity to oxidation and reduction as seen for the cell extracts. The high concentration (100 m*M*) of DTT did not reduce transcription in general since neither *bla* expression nor the basal level of *katG* expression was affected (Fig. 3B, lane 1 compared to lane 2). However, OxyR incubated in the presence of 100 m*M* DTT no longer activated expression of *katG* (Fig. 3B, lane 3 compared to lane 4). The inactivation of OxyR by 100 m*M* DTT was readily reversible since OxyR regained the ability to activate *katG* expression upon removal of the high concentration of DTT by gel filtration (Fig. 3B, lane 5). We also found that the same OxyR sample could then be reinactivated by 100 m*M* DTT and reactivated by removal of the DTT for a second time (Fig. 3B, lanes 6 and 7). These results suggest that the ability of OxyR to activate transcription is sensitive to the oxidation state of OxyR. The interconversion of OxyR between the active and inactive forms is also readily reversible within the 5-minute period required to remove the DTT.

The finding that DTT, a reductant, could reversibly inactivate the OxyR protein suggested that the redox state of the OxyR protein was coupled to its ability to stimulate transcription. However, the high concentrations of DTT might inhibit activation by denaturing OxyR or by chelating a metal required for function. To distinguish between these possibilities, we assayed the ability of OxyR to activate transcription under anaerobic conditions. In air-saturated buffers, OxyR is active in the presence of 10 m*M* DTT (Fig. 3C, lanes 1 and 2). If the transcription assays were performed in the presence of 10 m*M* DTT under a nitrogen atmosphere, OxyR no longer activated *katG* expression (Fig. 3C, lanes 3 and 4). These parallel assays (in which the only variable is the presence or absence of oxygen) strengthen our conclusion that the ability of OxyR to activate transcription is directly dependent on the state of OxyR oxidation.

DNA-binding of oxidized and reduced OxyR is distinct. Since we previously found that OxyR negatively autoregulates its own expression in vivo (*10*), we also examined the ability of OxyR to regulate *oxyR* transcription under oxidizing (1 m*M* DTT) and reducing (100 m*M* DTT) conditions with purified OxyR and RNA polymerase in vitro. The start of the *oxyR* transcript generated in vitro is consistent with the transcription start seen in vivo (*10*), and high levels of *oxyR* transcription are seen in the absence of OxyR (Fig. 4, lanes 1 and 3). The *oxyR* expression was reduced substantially in the presence of both reduced and oxidized OxyR (Fig. 4, lanes 2 and 4). The oxidized form of OxyR appears to be more efficient than the reduced form in repressing its own expression, in agreement with the observation that the synthesis of OxyR is decreased in cells treated with hydrogen peroxide compared to untreated cells (Fig. 1B).

We consistently see a decrease in the synthesis of the control *bla* gene in transcription reactions containing both the oxidized OxyR protein and the *oxyR* gene (Fig. 4, lane 2). This decreased expression of the *bla* gene is likely to be due to the titration of a limiting component in our transcription assays by the extremely high expression of a small RNA that is encoded in an orientation opposite to the *oxyR* gene and whose expression is highly induced by hydrogen peroxide in vivo (*21*). Repression by oxidized OxyR without a decrease in *bla* expression was observed in coupled transcription-translation assays in which all components are in greater excess (*18*).

The observation that reduced OxyR could act as a repressor suggested that OxyR was capable of binding to DNA under both oxidizing and reducing conditions. To investigate this possibility and further characterize the reduced form of OxyR, we assayed the ability of OxyR to bind to the *katG*, *ahpC*, and *oxyR* promoters

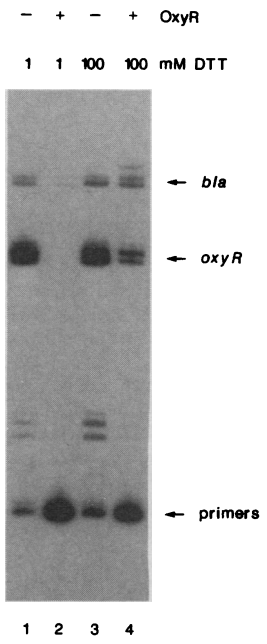

Fig. 4. Both oxidized and reduced OxyR repress *oxyR* expression in vitro. OxyR (1 μl of the centrifuged sample described in Fig. 3A) was assayed for the ability to repress transcription of the *oxyR* gene in the presence or absence of 100 m*M* DTT as described in Fig. 3B. A pUC12 plasmid (0.2 μg) carrying *oxyR* (pAQ17) (*10*) was used as the template. A distinct *bla* transcript extension product was visible on long exposures of lane 2, while no *oxyR* transcript extension product was detected.

under both conditions. DNase I assays in the presence of 1 and 100 m*M* DTT clearly show that OxyR binds to all three promoters, with high affinity under both oxidizing and reducing conditions (Fig. 5). Therefore, the conversion of reduced OxyR to a transcriptional activator upon oxidation is not likely due to significantly increased DNA-binding by the oxidized form of OxyR, but is probably due to a conformational change in OxyR already bound to the promoter regions.

In support of this conclusion, the footprints of oxidized and reduced OxyR are different. A distinct DNase I hypersensitive site (indicated by arrows in Fig. 5) is seen in the footprints with reduced-inactive but not oxidized-active OxyR. A DNase I hypersensitive site was also previously seen for IlvY (a member of the family of regulators showing homology to OxyR) in the absence but not in the presence of the inducer of the IlvY-mediated response (*22*). The lengths of the OxyR footprints at the *katG* and *oxyR* promoters are also different under oxidizing and reducing conditions (Fig. 5). While the footprint at the *katG* promoter is shortened in 100 m*M* DTT, the footprint at the *oxyR* promoter shows a clear extension under the reducing conditions. These observations suggest that the OxyR protein may change its contacts with the DNA. Although such alterations in footprint pattern are rare, *E. coli* RNA polymerase has also been found to drastically alter its contacts with DNA as the enzyme undergoes the transition from a closed to an open complex (*23*). The altered footprint of reduced OxyR is not an artifact due to the high concentrations of DTT since a noninducible OxyR mutant protein (resulting from the conversion of the cysteine residue at position 199 to serine), which no longer functions as an activator in vivo, shows a DNase I protection pattern in 1 m*M* DTT that is identical to the pattern seen for the wild-type protein in 100 m*M* DTT (*15*).

Oxidation as a mechanism for regulation. Our results show that OxyR, a transcriptional activator of genes induced by hydrogen peroxide, is activated directly by oxidation and is therefore both the sensor and transducer of an oxidative stress signal. We postulate that low amounts of reduced OxyR molecules are present in the cells at all times, bound to the promoters of *oxyR*-regulated genes. These OxyR molecules are poised to be activated by low amounts of hydrogen peroxide which presumably enter the cell by diffusion. Such a system allows for efficient and rapid induction of defense genes. Some of the other bacterial regulators that show homology to OxyR have also been found to be bound to the promoters that they regulate in both the presence and absence of inducer (*22, 24*) suggesting that members of this family of regulators may all be ideally designed for responses requiring immediate induction.

The differences in the footprints between reduced and oxidized OxyR at the *katG, ahpC*, and *oxyR* promoters suggest that a distinct conformational change in the OxyR protein is associated with the transition from the reduced (inactive) to the oxidized (active) state. Possibly the conformational change exposes an activation domain as has been proposed for numerous eukaryotic activators (*25*). Alternatively the conformational change may be associated with a change in oligomerization. Leucine residues that are spaced at intervals of seven amino acids reminiscent of a leucine zipper dimerization domain are highly conserved among the LysR-OxyR family of regulators (*15*). The details of how transcriptional activators contact polymerase to activate transcription are still largely unknown in both prokaryotes and eukaryotes. Hence, structural and genetic studies of the differences between the oxidized and reduced forms of OxyR should provide important insights into transcriptional activation in general.

The finding that the interactions of reduced OxyR with the individual promoters are different is also intriguing. Perhaps the different sequences at the three promoters dictate different conformational changes and roles for OxyR at the three promoters. At the *oxyR* promoter, OxyR activates transcription in one direction and represses transcription in the other, whereas OxyR probably only acts to induce transcription at the *katG* and *ahpC* promoters. Bölker and Kahmann have also characterized the binding of OxyR to the phage Mu *mom* promoter, which is repressed by OxyR (*11*). Interestingly, at this promoter, OxyR discriminates between methylated and unmethylated DNA (*11*). Further analysis of the specific DNA contacts made by reduced and oxidized OxyR at the different promoters should elucidate how OxyR is interacting with the different sequences and how a single protein can recognize several sequences that do not show apparent homology.

The nature of the oxygen species causing the conformational change and activation of OxyR in our assays has not yet been identified. Since OxyR is activated simply by the presence of air in 10 m*M* DTT in our in vitro assays, we suggest that reactive oxygen species are generated by the dissolved oxygen in our purification and assay buffers. Air-saturated solutions containing low concentrations of thiol compounds or detergents, both present in our buffers, are known to contain reactive oxygen species (*26*). We have prevented OxyR activation on removal of the 100 m*M* DTT by the addition of catalase (which breaks down hydrogen peroxide), suggesting that concentrations of hydrogen peroxide capable of activating OxyR are present under the aerobic conditions (*15*). Presumably, the rate of OxyR reduction by 100 m*M* DTT, but not 10 m*M* DTT, is sufficient to inhibit OxyR activation by the low concentrations of oxidants present in our buffer solutions.

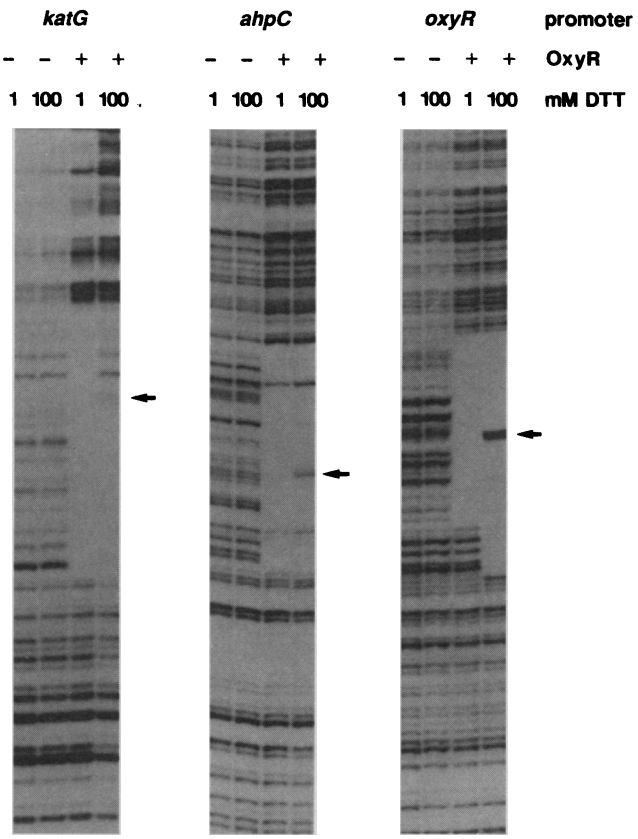

Fig. 5. Binding of oxidized and reduced OxyR to the *katG, ahpC*, and *oxyR* promoters. Samples of purified OxyR were incubated with *katG* (30 ng) (*13*), *ahpC* (30 ng) (*13*), and *oxyR* (7.5 ng) (*10*) promoter fragments in the presence of 1 and 100 m*M* DTT for 10 minutes. Subsequently, DNase I was added, and the samples were analyzed as described (*10, 13*). High concentrations of DTT do not alter the DNase I digestion patterns in the absence of OxyR.

Within the cell, OxyR is only a transcriptional activator for a limited time after oxidative stress since pulse-labeling experiments have shown that the induction of *oxyR*-regulated genes is predominant only during the first 10 minutes after treatment with hydrogen peroxide (*8*). However, the total levels of OxyR do not decrease significantly at any time point after treatment with the oxidant. Pulse-chase experiments also showed that the stability of the OxyR protein does not change after treatment with hydrogen peroxide (*15*). These observations suggest that bacterial cells possess a mechanism which allows for the inactivation of OxyR after a defined period of time, possibly by re-reduction of OxyR after the hydrogen peroxide is removed by catalase.

The nature of the redox-active center in OxyR is of interest. The candidates for this redox-active center are limited since the oxidation and reduction is readily reversible. Inter- or intra-molecular disulfide bonds meet this criterion, but we have never observed significant levels of a form of OxyR larger than 34 kD on immunoblots of non-reducing SDS gels probed with the OxyR antibody suggesting that no intermolecular disulfide bonds are formed (*18*). In addition, a mutant form of OxyR in which five of the six cysteine residues have been converted to serine shows wild-type induction of *oxyR*-regulated genes in vivo (*15*). It is also unlikely that OxyR is activated by the formation of an intermolecular disulfide bond with a small molecule, such as glutathione, since OxyR is activated by gel filtration that separates the protein from all small molecules in our assays. A cofactor or metal associated with OxyR might also be sensitive to oxidation; however, the addition of chelators did not effect the ability of OxyR to activate transcription (*15*). The one essential cysteine may also be reversibly oxidized to a sulfenic acid. Alternatively, OxyR may be activated by direct binding of the reactive oxygen species. Additional physical studies of OxyR should provide insights into how a protein can be oxidized in a very specific fashion.

The activities of several metabolic enzymes (*27*) and one other regulator of gene expression, the iron-responsive element (IRE) binding protein, have been found to be sensitive to oxidation in vitro (*28*). The IRE binding protein, a regulator of ferritin expression in human cells, requires free sulfhydryl groups for binding to IREs. This finding suggests that cells sense the presence or absence of iron, by modulating the reduction or oxidation of the IRE binding protein. It is likely that the activities of other regulators of gene expression are modulated by oxidation and reduction. OxyR may play a role in protecting the bacterial cell against the oxidative burst which is released when the bacterial cells encounter macrophages. Other prokaryotic and eukaryotic regulators may be activated by this oxidative burst or by the oxidants associated with degenerative diseases. Eye and lung tissue which are in contact with air may provide sources for identifying additional examples of redox-sensitive regulators. Regulators in chloroplasts and mitochondria, the sites of electron transport associated with photosynthesis and oxidative phosphorylation, may also be sensitive to oxidation or reduction, and the redox state of additional regulators may be coupled to the presence or absence of metals. Whether oxidation-reduction is a general mechanism of regulation remains to be determined as additional signal transduction pathways are characterized.

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    katG 5'-ATGTAAGATCTCAACTATCGCATCCGTGGATTAATTCAATTATAA-3'

    ahpC 5'-GGGTTGTTAGTTAACGCTTATTGATTTGATAATGGAAACGCATTAG-3'

    oxyR 5'-GCCACGATAGTTCATGGCGATAGGTAGAATAGCAATGAACGATTA-3'

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