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Mutational Analysis of the Redox-Sensitive Transcriptional Regulator OxyR: Regions Important for Oxidation and Transcriptional Activation

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OxyR is a redox-sensitive transcriptional regulator of the LysR family which activates the expression of genes important for the defense against hydrogen peroxide in *Escherichia coli* and *Salmonella typhimurium*. OxyR is sensitive to oxidation and reduction, and only oxidized OxyR is able to activate transcription of its target genes. Using site-directed mutagenesis, we found that one cysteine residue (C-199) is critical for the redox sensitivity of OxyR, and a C-199→S mutation appears to lock the OxyR protein in the reduced form. We also used a random mutagenesis approach to isolate eight constitutively active mutants. All of the mutations are located in the C-terminal half of the protein, and four of the mutations map near the critical C-199 residue. In vivo as well as in vitro transcription experiments showed that the constitutive mutant proteins were able to activate transcription under both oxidizing and reducing conditions, and DNase I footprints showed that this activation is due to the ability of the mutant proteins to induce cooperative binding of RNA polymerase. Unexpectedly, RNA polymerase was also found to reciprocally affect OxyR binding.

The OxyR protein is a transcriptional activator of genes important for the defense against oxidative stress in *Escherichia coli* and *Salmonella typhimurium* (14, 37). Upon exposure to hydrogen peroxide, OxyR induces the expression of several genes, including *katG* (encoding HPI catalase), *ahpCF* (encoding an alkyl hydroperoxide reductase), *dps* (encoding a nonspecific DNA-binding protein), *gorA* (encoding glutathione reductase), and *oxyS* (encoding a small untranslated, regulatory RNA), and the cells become more resistant to oxidative stress (3, 4, 39, 41). During normal growth, OxyR also acts as a repressor, negatively autoregulating its own expression and the expression of the Mu phage *mom* gene (6, 14, 38). Here and in the accompanying paper (20), we identify regions of the OxyR protein critical for activation and repression.

OxyR is 34 kDa in size and belongs to the LysR-type family of transcriptional regulators (6, 14, 37, 43). LysR family members are all DNA-binding proteins which positively regulate expression of their target genes, and many of the regulators also repress their own expression (reviewed in reference 29). The ability of most LysR-type proteins to activate transcription is dependent on the presence of a coinducer, such as octopine for the *Agrobacterium tumefaciens* OccR protein (42), indoleglycerol phosphate for *Pseudomonas aeruginosa* TrpI (12), *N*-acetylserine for *E. coli* CysB (26), and flavonoids for the NodD proteins of different species of *Rhizobium* (reviewed in reference 16). The central region of the LysR-type proteins is assumed to be involved in coinducer binding, since *Rhizobium leguminosarum* NodD and *Pseudomonas putida* NahR mutations which caused an altered response to the inducer mapped to the central domain (9, 18, 24, 30), and an exchange between the central domains of NodD proteins from different *Rhizobium* species leads to a different coinducer specificity of the resulting hybrid proteins (31). The fact that members of the LysR-type family of transcriptional regulators have only little homology in this protein region probably reflects the variety of coinducers to which these proteins respond. Unlike many of the other LysR-type regulators, the OxyR protein does not appear to bind a coinducer but rather is activated by direct oxidation. In vitro transcription experiments with purified components showed that the oxidized but not the reduced form of OxyR was able to activate transcription (35), but the redox-active center has not been characterized.

A transcriptional activator may increase RNA polymerase binding, open complex formation, or promoter clearance (reviewed in reference 2). A few studies suggest that LysR-type regulators contact the α subunit of RNA polymerase and act to increase polymerase binding to the promoter. OxyR binds adjacent to the −35 sequence of the positively regulated promoters, and Tao et al. have shown that OxyR acts cooperatively to increase RNA polymerase binding to the *katG* promoter (36). Mutations in the α subunit of polymerase, such as a change of R-265 to C (R265C), prevented activation by OxyR (36). Similarly, mutations at codon 271 of the α subunit of RNA polymerase prevent activation by the LysR-type regulator CysB (40). The domains in OxyR and other LysR family members that touch RNA polymerase have not been identified.

In recent studies on the DNA binding properties of OxyR, we found that the reduced and oxidized (activated) forms of OxyR require different DNA contacts for binding (41). Oxidized OxyR binds in four adjacent major grooves, while reduced OxyR contacts two pairs of adjacent major grooves separated by one helical turn. The reduced protein has significantly reduced affinities for the *katG* and *ahpCF* promoters, showing that only a subset of the OxyR-regulated promoters

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carry determinants for the binding of reduced OxyR. The two binding modes probably allow OxyR to repress the *oxyR* and *mom* promoters during normal growth, while activating *katG*, *ahpCF*, *dps*, *gorA*, and *oxyS* only upon oxidative stress. These studies also suggest that the conformations of reduced and oxidized OxyR are significantly different.

In the present study, we used site-directed as well as random mutagenesis to characterize the domains of the OxyR protein required to sense oxidative stress and affect transcription initiation. The six possible redox-reactive cysteines in OxyR were mutagenized to serine, and a single cysteine (Cys-199) was found to be critical for activity. Random mutagenesis revealed that a region around this critical cysteine is involved in transcriptional activation, since several mutations causing the constitutive phenotype mapped to this region. We were also able to show that the ability of mutants to activate transcription correlates with their ability to induce RNA polymerase binding. Moreover, DNase I footprinting assays demonstrated that while OxyR induces polymerase binding, RNA polymerase also reciprocally influences OxyR binding.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this study are described in Table 1. Strain GSO5 was constructed as follows. The 0.2-kb *Hin*dII-*Ssp*I fragment of pAQ17 (14), carrying the *oxyR-oxyS* promoter region, was cloned into the unique *Sma*I site of pTS7 (1) to create the *galK* fusion. The plasmid was then recombined onto λ_{Y2055} (*c*I857 *galK nin*5) and integrated into the *att*λ site of SA2692 (1). A Δ*oxyR*::*kan* deletion-insertion mutation (3) was subsequently moved into the strain by P1 transduction. pAQ5 (34) was used for the random mutagenesis and is a pACYC184 derivative which carries *oxyR* on a *Bam*HI-*Eco*RV fragment. Since these pACYC184-derived plasmids did not prove to be suitable for double-strand sequencing, the *Bam*HI-*Hin*dIII *oxyR* fragments were subcloned into M13mp18 for sequencing. For overproduction of the mutant OxyR proteins, *oxyR* was cloned behind the P_{tac} promoter of pKK177-3 as follows. First, the *Ssp*I-*Hin*dIII fragment of wild-type *oxyR* in pUC18 carrying the modified Shine-Dalgarno sequence (35) was replaced with the same fragment of the mutant *oxyR* genes from pACYC184. These constructs were sequenced, and subsequently the *Eco*RI-*Hin*dIII fragment was subcloned into pKK177-3. All sequencing and subcloning were carried out by standard procedures.

Media and growth conditions. Strains were grown in LB medium (25), and ampicillin (100 μg/ml [final concentration]), kanamycin (25 μg/ml), chloramphenicol (25 μg/ml), or tetracycline (15 μg/ml) was added when appropriate. For overproduction of the mutant proteins, the cells were grown in TB medium (28). The resistance of strains to hydrogen peroxide and cumene hydroperoxide was assayed by zones of inhibition, which were determined as described previously (13) except that the strains were grown in and plated on LB medium containing the appropriate antibiotics.

Mutagenesis. Site-directed mutagenesis was carried out with an oligonucleotide-directed in vitro mutagenesis system (Amersham, Arlington Heights, Ill.). The base pair substitutions that were introduced are listed in Table 2. Random mutagenesis with hydroxylamine was carried out with approximately 10 μg of purified pAQ5 plasmid DNA as described previously (15). The DNA (20 μl) was mixed with 100 μl of a 0.5 M potassium phosphate–5 mM EDTA solution (pH 6) and 80 μl of a freshly prepared 1 M hydroxylamine solution (pH 6) and incubated for 60 min at 65°C. The samples were dialyzed extensively against Tris-EDTA buffer and used directly (5- to 10-μl aliquots) to transform *E. coli* XL1-blue cells. The XL1-blue transformants were rinsed off the plates, and the plasmid DNA was reisolated and then used to transform the GSO5 recipient strain. Finally, the GSO5 transformants were screened for the desired phenotype on MacConkey agar plates.

Protein overexpression and purification. For overproduction of the mutant OxyR proteins, *oxyR* deletion strains (TA4484) with the corresponding pKK177-3 derivatives were grown to mid-exponential phase and then induced with 250 μg of IPTG (isopropyl-β-D-thiogalactopyranoside) per ml for 2 h. The cells were lysed by several passages through a French pressure cell, and the insoluble fraction was removed by centrifugation. The soluble fraction was then applied to a heparin-Sepharose column (Pharmacia, Piscataway, N.J.). The purification procedure was carried out as described previously (35) except that the buffer Z contained 50 mM HEPES (*N*-2-hydroxypiperazine-*N*′-2-ethanesulfonic acid) (pH 8), 5 mM $MgCl_2$, 0.5 mM EDTA (pH 8), and 10% (vol/vol) glycerol. The A233V and E225K mutants were eluted with a 0.1 to 0.4 M KCl gradient instead of the second wash with buffer Z containing 0.2 M KCl. Both the A233V and E225K mutants eluted at about 0.2 M KCl. Aliquots of the peak fractions were analyzed on sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS-PAGE) gels, using the buffer system of Laemmli (21). The protein concentration in all of the preparations was approximately 0.8 mg/ml, and roughly 8 mg of approximately 90% pure OxyR protein was obtained from a 1-liter cell culture.

TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
XL1-blue	F′ [*proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15* Tn*10* (Tc^r)]	8
D1210	HB101/F′ [*lacI*^{q}]	5
TA4112	*oxyR*Δ*3*	13
TA4484	*oxyR*Δ*3*, pMC7	39
SA2692	HB101 *recA*$^+$ Δ*lac* Δ*gal-165*	1
GSO5	SA2692 Δ*oxyR*::*kan* (λ_{Y2055} *oxyS-galK*)	This study
Plasmids		
pTS7	pBR322 *int* P′P; promoterless *lacZ* and *galETK*, Ap^r	1
pMC7	*lacI*^{q} Tc^r	10
pACYC184	Cm^r Tc^r	11
pKK177-3	P_{tac} promoter, derivative of pKK223-3, Ap^r	7
pAQ5	*oxyR* wt[a] in pACYC184, Cm^r, Ap^r	34
pAQ17	*oxyR* wt in pUC12	14
pAQ25	*oxyR* wt in pKK177-3 with altered SD[b] sequence, Ap^r	35
pGSO8	*oxyR* C25S in pUC18	This study
pGSO9	*oxyR* C143S in pUC18	This study
pGSO10	*oxyR* C180S in pUC18	This study
pGSO11	*oxyR* C208S in pUC18	This study
pGSO12	*oxyR* C259S in pUC18	This study
pGSO13	*oxyR* 5CS[c] in pUC18	This study
pGSO51	*oxyR* T100I in pACYC184	This study
pGSO52	*oxyR* H114Y in pACYC184	This study
pGSO53	*oxyR* H198Y in pACYC184	This study
pGSO54	*oxyR* H198R in pACYC184	This study
pGSO55	*oxyR* R201C in pACYC184	This study
pGSO56	*oxyR* C208Y in pACYC184	This study
pGSO58	*oxyR* A233V in pACYC184	This study
pGSO59	*oxyR* A233T in pACYC184	This study
pGSO60	*oxyR* G253K in pACYC184	This study
pGSO66	*oxyR* E225K in pACYC184	20
pGSO67	*oxyR* H198R in pKK177-3	This study
pGSO68	*oxyR* C199S in pKK177-3	This study
pGSO69	*oxyR* A233V in pKK177-3	This study
pGSO70	*oxyR* E225K in pKK177-3	This study
pGSO71	*oxyR* S33N in pKK177-3	This study
pGSO72	*oxyR* C199S in pUC18	This study
pGSO73	*oxyR* C199S in pACYC184	This study

[a] wt, wild type.
[b] SD, Shine-Dalgarno.
[c] Cysteine residues 25, 143, 180, 208, and 259 changed to serine.

Primer extension assays. Cells were grown to an optical density at 600 nm of 0.4, and then half of each sample was treated with hydrogen peroxide (200 μM final concentration) for 10 min. Total RNA was isolated by using hot phenol, and 0.1 pmol of an end-labeled *oxyS* oligonucleotide (5′-GCAAAAGTTCACGT TGG) was annealed to 3 μg of total RNA as described previously (33 [short protocol]). The extension reaction was performed with Superscript reverse transcriptase from Gibco BRL (Gaithersburg, Md.) in the reaction buffer provided. The extension products were separated on an 8% sequencing gel and were quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

DNase I footprinting assays. An end-labeled DNA fragment (20,000 cpm) was incubated with 1 to 2 μl of the soluble fraction of a crude cell extract or with 10 to 100 ng of pure OxyR protein in 25 μl of 0.5× TM buffer (39). Aliquots (6 pmol) of RNA polymerase (Pharmacia) were added to the binding reaction mixtures when indicated. The binding reaction mixtures were then treated with DNase I as described previously (39). The crude cell lysates were prepared by sonicating the pellet of a 5-ml overnight culture in 800 μl of 10 mM Tris buffer (pH 8) containing 20% glycerol. The soluble fraction was obtained by centrifugation.

In vitro transcription assays. The transcription assays were carried out at 37°C as described previously (35). Purified OxyR (5 pmol) was first incubated with

TABLE 2. Sensitivities of *oxyR* cysteine-to-serine mutants to oxidants

Strain	Codon exchange	Zone(s) of inhibition[a] (mm) with:		Phenotype[b]
		10% H_2O_2	4% CHP	
Vector (pUC18)		36	27	
Wild type		24 (29)	18	
C25S	TGC→TCC	24 (29)	19	Wild type
C143S	TGC→TCC	24 (29)	18	Wild type
C180S	TGC→TCC	24 (29)	18	Wild type
C199S	TGT→TCT	40	26	Noninducible
C208S	TGT→TCT	29	18	Wild type
C259S	TGC→TCC	25 (31)	17	Wild type
5CS[c]		29	17	Wild type

[a] Total diameter of the growth inhibition zone caused by the addition of hydrogen peroxide (H_2O_2) or cumene hydroperoxide (CHP). The values are from a representative assay. The numbers in parentheses indicate the size of the second zone of partial growth.

[b] Phenotype of mutants with respect to their sensitivity to oxidants.

[c] In this mutant, cysteine residues 25, 143, 180, 208, and 259 were changed to serine.

pAQ17 (0.2 μg) for 10 min, and then RNA polymerase (6 pmol) was added and the reaction mixtures were incubated for another 10 min. After the addition of 1 μl of a 25 mM nucleoside triphosphate mixture, the reaction mixtures were incubated for an additional 5 min, and then the reactions were terminated by several phenol extractions. One-third of each sample was analyzed by primer extension with end-labeled *oxyS* (5′-GCAAAAGTTCACGTTGG) and *bla* (5′-AGGGCGACACGGAAATGTTGAATACTCATA) oligonucleotide primers. The primer extension assays were carried out as described above with the exception that after the extension reaction, the samples were treated with 5 μg of RNase A and subjected to a phenol extraction and ethanol precipitation.

RESULTS

Mutagenesis of cysteine residues. Previous studies have shown that only oxidized OxyR is able to activate transcription (35). To determine whether the cysteine residues in OxyR were important for sensing the oxidative stress signal, we changed each of the six cysteines to serine by site-directed mutagenesis. The mutants were cloned into pUC18 and transformed into TA4112, an *E. coli* strain carrying a chromosomal deletion of *oxyR*. These strains were then assayed for their sensitivities to hydrogen peroxide and cumene hydroperoxide by using a growth inhibition assay. As seen in Table 2, the C25S, C143S, C180S, and C259S mutant strains showed the same sensitivity as a strain carrying the wild-type *oxyR* gene. The strains showed two distinct zones of inhibition for hydrogen peroxide; the first zone corresponded to complete killing, while the second zone corresponded to partial growth. The reason for this double zone is unclear, but the C208S mutant, although resistant, showed only one zone of inhibition for hydrogen peroxide. Unlike the C25S, C143S, C180S, C208S, and C259S mutants, the C199S mutant strain was as hypersensitive to hydrogen peroxide and cumene hydroperoxide as the control strain lacking *oxyR*, suggesting that the C199S mutant was unable to induce the expression of defense genes. The C199S protein was stable and able to specifically bind DNA (see below). Therefore, we propose that the C-199 residue is the possible redox center and that the C199S mutant is locked in the reduced conformation. A mutant in which all cysteine residues except C-199 were replaced by serine showed the same resistance as the C208S single mutant.

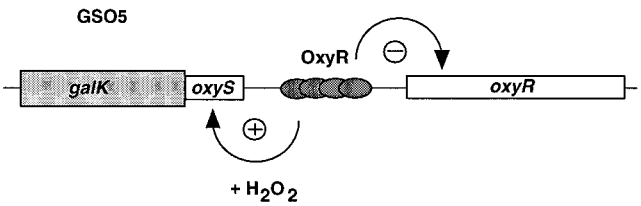

FIG. 1. *oxyS-galK* transcriptional fusion used to screen for OxyR mutants. When binding to the promoter region, OxyR represses its own expression, and upon oxidation, it activates *oxyS* transcription.

Screen for constitutively active OxyR mutants. Having identified C-199 as a critical amino acid for OxyR activity, we wanted to determine what other amino acids were required for OxyR to act as an activator. We chose to randomly mutagenize the entire *oxyR* gene and screen for OxyR mutants altered in their ability to activate transcription of the *oxyS* promoter. The *oxyS* gene encodes a small, untranslated regulatory RNA and is encoded divergently from the *oxyR* structural gene (4). The *oxyR* and *oxyS* promoters overlap, and OxyR bound to its site at these promoters acts to both repress *oxyR* expression (under oxidizing and reducing conditions) and activate *oxyS* expression (only under oxidizing conditions). We constructed a transcriptional fusion between the *oxyS* promoter and the *galK* reporter gene and integrated this fusion into the chromosome of *E. coli* SA2692 (Fig. 1). Subsequently, an *oxyR* deletion was moved into the strain by P1 transduction, giving rise to GSO5. Plasmids carrying *oxyR* were then mutagenized by hydroxylamine in vitro and introduced into the GSO5 background.

The abilities of the *oxyR* mutants to activate *oxyS* expression were monitored on MacConkey agar plates with galactose as a carbon source. For reasons that are not understood, wild-type OxyR displayed an uninducible or reduced phenotype on the MacConkey-galactose medium, even in the presence of hydrogen peroxide. The uninduced phenotype, however, suggested the possibility of screening for constitutively active mutants on this medium. We screened more than 10^6 colonies for constitutively active mutants and identified 20 candidates. The mutants were tested for their sensitivities to hydrogen peroxide and cumene hydroperoxide in a growth inhibition assay, and 18 of the mutants showed smaller zones than the wild-type strain, indicating that they were more resistant to oxidants. Only the A233V mutant consistently had the two zones of growth inhibition seen for some of the cysteine mutants. Unlike the result shown in Table 2, only a single zone was observed for the strain expressing wild-type OxyR, probably because of differences in the strain backgrounds and vectors.

Mutations map to the C-terminal part of OxyR. To determine the locations and natures of the mutations, the mutant *oxyR* genes were sequenced entirely. Eight different mutations, all causing single amino acid exchanges, were found in the pool of 18 resistant candidates (Table 3). The A233T mutation, which caused a constitutive phenotype in our random screen, affected the same alanine residue mutated (A233V) in the original *oxyR2* constitutive mutant strain (14). Similarily, two different amino acid changes at position H-198 gave rise to a constitutive phenotype.

The positions of the mutations causing the constitutive phenotype are shown on an alignment of OxyR and five other members of the LysR family in Fig. 2. The location of the noninducible C199S mutation is also indicated in Fig. 2. All of the mutations are clustered in three regions in the C-terminal two-thirds of the protein, suggesting that these regions are

TABLE 3. Sensitivities of constitutively active *oxyR* mutant strains to oxidants

Strain	Codon exchange	Zone(s) of inhibition[a] (mm) with:		Phenotype[b]
		10% H_2O_2	4% CHP	
Vector (pACYC184)		35	23	
Wild type		24	20	
C199S	TGT→TCT	42	30	Noninducible
T100I	ACA→ATA	24	12	Constitutive
H114Y	CAC→TAC	21	14	Constitutive
H198Y	CAC→TAC	21	13	Constitutive
H198R	CAC→CGC	23	14	Constitutive
R201C	CGC→TGC	23	16	Constitutive
C208Y	TGT→TAT	23	13	Constitutive
A233V[c]	GCA→GTA	21 (27)	13	Constitutive
A233T	GCA→ACA	16	12	Constitutive
G253K	GGG→AAG	19	13	Constitutive

[a] Total diameter of the growth inhibition zone caused by the addition of hydrogen peroxide (H_2O_2) or cumene hydroperoxide (CHP). The values are averages from two separate assays. The number in parentheses indicates the size of the second zone of partial growth.

[b] Phenotype of mutants with respect to their color on MacConkey agar plates.

[c] This mutant corresponds to the previously isolated *oxyR2* mutant (14).

involved in sensing oxidative stress and transducing the signal to RNA polymerase. One cluster of mutations affects residues T-100 and H-114, another cluster is evident around the critical cysteine C-199, and a third is located further downstream at positions A-233 and G-253, which are at and near the original *oxyR2* mutation (A233V).

Mutants show constitutive *oxyS* transcription in vivo. The phenotype of the constitutive mutants on MacConkey agar plates suggested that, in contrast to the case for the wild-type protein, oxidation was not required for the constitutive mutants to activate *oxyS* transcription. To assess whether oxidation would increase the activities of the mutants and to compare the mutants with the wild-type protein in vivo, we divided cultures expressing the different *oxyR* mutants and treated half of each culture with hydrogen peroxide. Total RNA was then isolated from both the treated and untreated cells, and the levels of the *oxyS* message were determined by primer extension. Unlike the wild-type strain, all of the untreated constitutive mutants showed *oxyS* expression (Fig. 3). Only two of the mutants (T100I and H114Y) showed a two- to threefold induction of *oxyS* expression upon treatment with hydrogen peroxide. Although all of the constitutive mutants were active under reducing conditions, the overall level of activity varied from 3 to 400% of the wild-type activity. The noninducible C199S mutant did not show any activation of *oxyS*. The hydrogen peroxide-induced expression of two additional OxyR-regulated genes, *dps* and *katG*, was also tested for a subset of the mutants (H198Y, C199S, and A233V). All of the mutants showed the same relative expression seen with the *oxyS* gene, suggesting that the individual OxyR mutants had similar effects on all target genes (data not shown).

DNA binding by OxyR constitutive mutants. We assumed that OxyR binding to its target promoters is required for transcriptional activation. Previous studies had also shown that the oxidized and the reduced forms of OxyR have different DNA

FIG. 2. Protein sequence alignment of six LysR family members and locations of the OxyR mutations causing a constitutively active phenotype. Residues that are identical in four of six sequences are in boldface, the bars mark the helix-turn-helix motif, and the six cysteine residues in OxyR are underlined. The x denotes the noninducible C199S mutant. The sequences were obtained from SWISS-PROT and GenBank (*E. coli* OxyR [OXYR_ECOLI], *E. coli* CysB [CYSB_ECOLI], *P. putida* NarR [NAHR_PSEPU], *Rhizobium meliloti* NodD1 [NOD1_RHIME], *P. aeruginosa* TrpI [TRPI_PSEAE], and *A. tumefaciens* OccR [TIPOCCR]). The alignment was done with the Genetics Computer Group program PILEUP with default parameters.

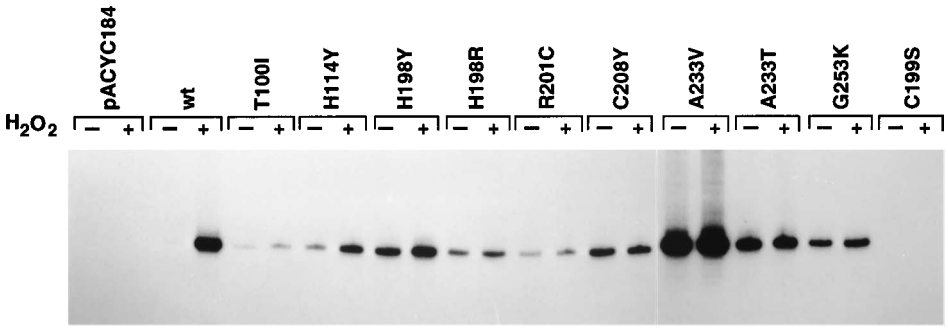

FIG. 3. Primer extension assays of *oxyS* induction in OxyR constitutive mutant strains. Total RNA was isolated from the corresponding *E. coli* strains, which were either untreated (−) or induced with 200 μM hydrogen peroxide for 10 min (+). A labeled oligonucleotide capable of hybridizing to the *oxyS* transcript was then incubated with 3 μg of total RNA and extended with reverse transcriptase. wt, wild type.

binding characteristics (35, 41). For example, at the *oxyR-oxyS* promoter, the reduced protein has a DNase I footprint that is extended compared with that of the oxidized protein. The reduced, but not the oxidized, footprint also shows a strong central DNase I-hypersensitive site. Therefore, to further characterize the mutants, we prepared cell extracts from Δ*oxyR*::*kan* strains expressing the wild-type and mutant OxyR proteins from pACYC184. We then assayed the extracts for binding to an *oxyR-oxyS* promoter fragment. If the mutations caused the proteins to be locked in the oxidized conformation, we should observe the shorter DNase I footprint characteristic of the oxidized wild-type protein. Alternatively, if the constitutive phenotype was caused only by the abnormal exposure of an activation domain, the mutants should bind in the extended conformation characteristic of reduced wild-type OxyR.

Since OxyR present in extracts prepared aerobically in the absence of high concentrations of dithiothreitol (DTT) is predominantly in the oxidized form (35), the lysate from wild-type cells gave rise to a short footprint (Fig. 4A). Among the constitutive mutants however, only G253K showed the shorter footprint. Four other constitutive mutants (H198Y, H198R, R201C, and C208Y) showed the extended DNase I footprint observed with the reduced wild-type protein. The extension was seen even when the extracts were treated with 0.2 or 2 mM hydrogen peroxide (data not shown). These results suggested that the H198Y, H198R, R201C, and C208Y proteins bind in the reduced configuration under oxidizing conditions. Reduced wild-type OxyR is not able to bind to the *katG* and *ahpC* promoter fragments, since these promoters lack determinants for reduced OxyR binding (41), and although H198R constitutively activates the *katG* and *ahpC* genes, we observed that the purified mutant protein did not give a footprint at either of these promoters (data not shown). However, the binding of RNA polymerase was found to alter the binding of H198R to the *oxyR-oxyS* fragment (see below), and an interaction with RNA polymerase may allow the H198R protein to bind to and constitutively activate the *katG* and *ahpC* promoters.

Unexpectedly, four constitutive mutants (T100I, H114Y, A233V, and A233T) behaved like E225K, a nonbinding mutant (described in reference 20), and did not show a distinct footprint even though equivalent amounts of OxyR protein were present in the samples (Fig. 4A and data not shown). Since the extracts for both the A233V and A233T mutants caused nonspecific degradation of the *oxyR-oxyS* probe, we repeated the DNase I footprint experiment with purified A233V but still did not observe a footprint (see below). These binding studies demonstrated that T100I, H114Y, A233V, and A233T have decreased apparent affinities to DNA. We did detect a weak shifted band for all four mutants in a more sensitive gel retardation assay, revealing some residual DNA binding activity which may be sufficient for activation of *oxyS* (data not shown). In addition, this weak binding must allow for autorepression by the mutant proteins, since none of the constitutively active mutants showed elevated levels of OxyR protein by immunoblot assays (data not shown). As shown below, the DNA binding affinities of some of the mutants may also be increased by the binding of RNA polymerase.

We also characterized the DNase I footprints of all the cysteine mutants created by site-directed mutagenesis (Fig. 4B). The C25S, C143S, C180S, and C259S mutants showed a short footprint, while the C199S and C208S mutants gave an extended footprint with the hypersensitive cleavage site seen with the reduced wild-type protein. The C208S mutant also showed a slightly decreased binding to the DNA compared with the wild-type protein and the other C-to-S mutants.

Transcriptional activation by mutant OxyR proteins in vitro. To study some of the different OxyR mutants in vitro, we overexpressed and purified the noninducible C199S protein and two representative constitutive mutant proteins, H198R and A233V (Fig. 5). We also overexpressed and purified two nonbinding mutants, S33N and E225K (described in the accompanying paper [20]). We then assayed the abilities of the purified wild-type and mutant proteins to activate transcription in vitro. Aliquots of the purified proteins were incubated with a supercoiled plasmid carrying the *oxyS* and *bla* (conferring ampicillin resistance) genes together with purified RNA polymerase holoenzyme in a transcription reaction. Subsequently, the in vitro-synthesized *oxyS* and *bla* transcripts were detected by primer extension assays (Fig. 6). A distinct OxyR-dependent signal was observed for *oxyS* with the wild-type protein (Fig. 6A), since aerobically purified wild-type OxyR is predominantly oxidized (35). We did not detect an *oxyS* transcript with C199S, consistent with our observations that this mutant was unable to activate transcription in vivo. The constitutive H198R mutant was nearly as active as the wild-type protein, but the constitutive A233V protein showed significantly less activity than either wild-type OxyR or H198R. The low level of activity of the A233V mutant in vitro was surprising, since this mutant is the strongest activator in vivo. Possibly the A233V protein is altered during purification or requires additional factors for stabilization (discussed in reference 20). The nonbinding mutant E225K showed very weak activity, while the second nonbinding mutant, S33N, did not show any detectable activity. The expression of the control *bla* gene was unaffected by the different OxyR mutants.

To compare the activities of the wild-type protein and the

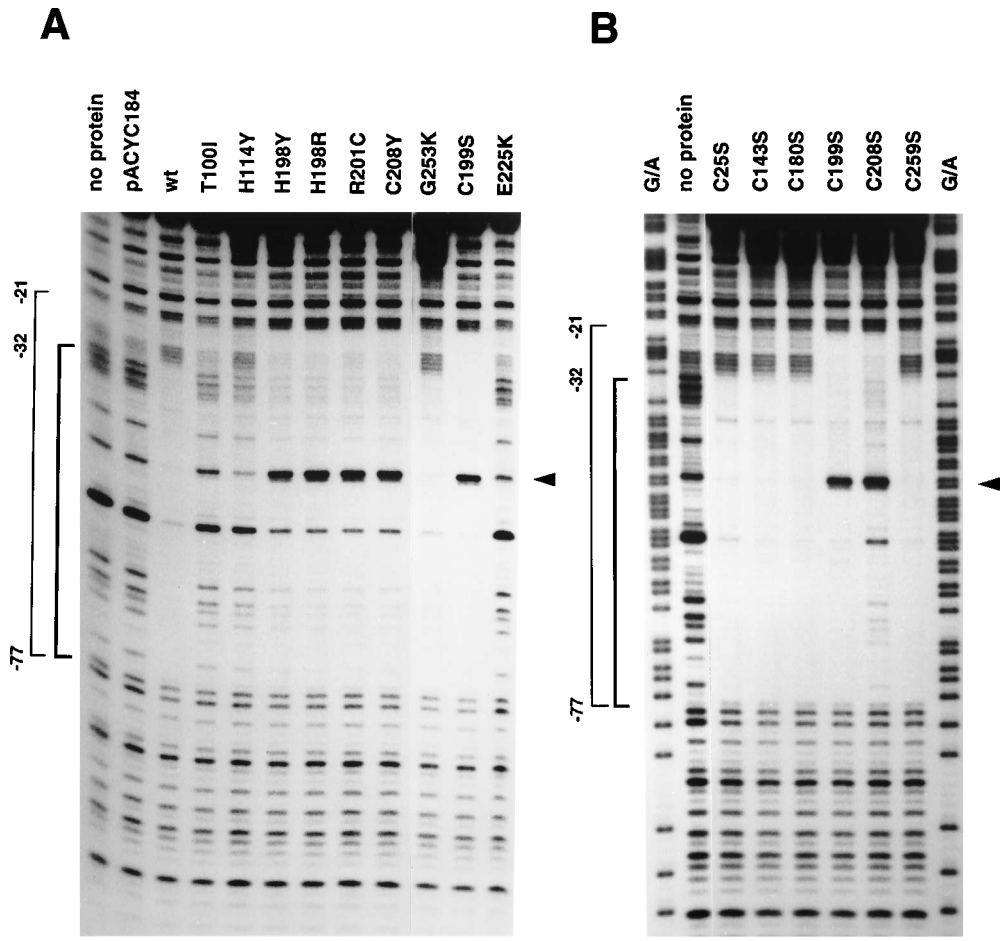

FIG. 4. DNase I footprints of the OxyR mutants binding to the top strand of the *oxyS* promoter. The 100-bp *Eco*RI-*Hin*dIII fragment of pGSO40 (41) labeled at the *Hin*dIII site was incubated with 2 μl of the soluble fraction of crude cell extracts. (A) Constitutive *oxyR* mutations carried on pACYC184; (B) cysteine mutations carried on pUC18. The heavy bracket indicates the oxidized shorter footprint of OxyR, the light bracket indicates the reduced extended footprint of OxyR, and the arrowhead indicates the hypersensitive cleavage site seen with the extended footprint. The positions of the footprints are labeled with respect to the start of the *oxyS* transcript, and the G/A sequence of the fragment is shown in panel B. wt, wild type.

constitutive mutant H198R protein under reducing conditions, the in vitro transcription assays were also carried out in presence of 200 mM DTT (Fig. 6B). For the wild-type protein, the activity under reducing conditions dropped to 12% of the activity in the absence of DTT, while for the H198R mutant, the activity was only reduced to 48% of the activity in the absence of DTT, showing that the H198R protein is less sensitive to reducing conditions. These observations agree with our findings that H198R is active under normal, reducing conditions in vivo.

Cooperative binding between mutant OxyR proteins and RNA polymerase. Tao and colleagues have shown that OxyR has a cooperative effect on RNA polymerase binding to the *katG* promoter site (36). To test whether the mutant proteins affected polymerase binding, we analyzed the DNase I footprint of RNA polymerase binding to the *oxyR-oxyS* promoter fragment in the presence of the purified OxyR mutants (Fig. 7A). RNA polymerase (0.3 to 10 pmol) alone did not show a footprint at the *oxyS* promoter (Fig. 7A, lane 2). However, when the polymerase was incubated with oxidized wild-type OxyR, a clear protection due to polymerase was observed at the *oxyS* promoter (Fig. 7A, lane 4). The uninducible mutant, C199S, showed an extended footprint alone (Fig. 7A, lane 5)

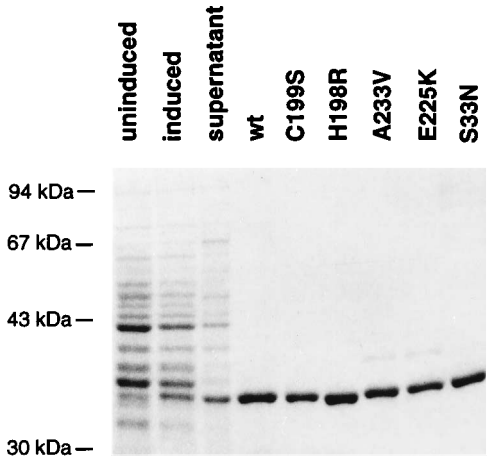

FIG. 5. Overexpression and purification of OxyR mutant proteins. Aliquots of uninduced and induced cultures, the soluble fraction of induced cells (described in Materials and Methods), and purified protein fractions (80 to 100 μg) were electrophoresed on an SDS–12% PAGE gel and stained with Coomassie blue. The A233V and E225K proteins showed reduced affinities to the heparin and eluted at lower salt concentrations than the wild-type protein, which likely accounts for the contaminating protein band in these samples. The mobilities of the standard proteins are indicated to the left of the gel. wt, wild type.

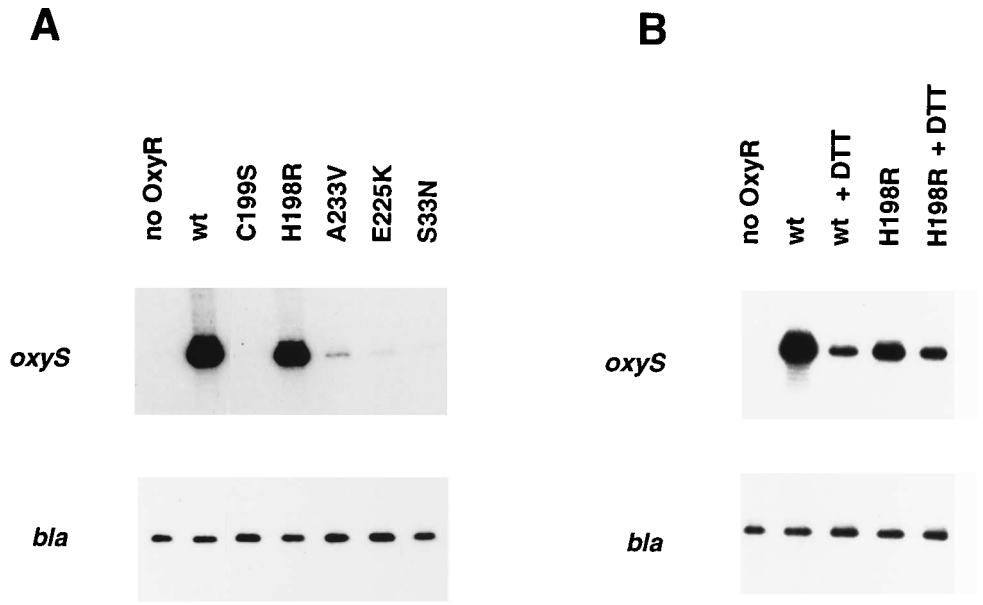

FIG. 6. In vitro transcription assays of purified wild-type (wt) and OxyR mutant proteins. The transcription reactions were carried out as described in Materials and Methods. The transcripts were then detected with *oxyS*- and *bla*-specific oligonucleotides in a primer extension assay. (A) Assays with the purified wild-type and C199S, H198R, A233V, E225K, and S33N mutant proteins under oxidizing conditions (no DTT); (B) assays with the wild-type and constitutive H198R mutant proteins in the absence or presence of 200 mM DTT.

and did not induce RNA polymerase binding (lane 6), consistent with the lack of transcription activation seen with this mutant. In contrast, the constitutive H198R mutant, which also showed an extended footprint alone (Fig. 7A, lane 7), had a cooperative effect on polymerase binding (lane 8). Interestingly, the strong DNase I-hypersensitive site detected for H198R alone was not seen in the presence of polymerase. The A233V constitutive mutant, which did not bind to the DNA alone (Fig. 7A, lane 9), nevertheless stimulated RNA polymerase binding (lane 10), although the binding was weaker than that observed with H198R. The nonrepressing mutants E225K and S33N (described in the accompanying paper [20]) also did not bind to DNA alone (Fig. 7A, lanes 11 and 13), but E225K could still induce some cooperative binding of RNA polymerase (lane 12). These footprints showed that the constitutive mutants (H198R and A233V), but not the noninducible mutant (C199S), had a cooperative effect on RNA polymerase binding to the *oxyS* promoter. The studies also showed that an interaction with RNA polymerase increased the DNA binding affinity of the A233V and E225K mutant proteins.

We examined RNA polymerase binding with wild-type OxyR and H198R in the presence of 200 mM DTT (Fig. 7B). The wild-type protein did not increase the binding of polymerase under the reducing conditions (Fig. 7B, lane 7), while the constitutively active H198R mutant still had a cooperative effect on polymerase in the presence of the 200 mM DTT (lane 11). These observations support the conclusion that H198R is constitutively active and that the ability to induce RNA polymerase-promoter binding correlates with the ability to activate transcription.

DISCUSSION

In this study, we used site-directed and random mutageneses to isolate one noninducible and eight constitutively active OxyR mutants. Together with the nonbinding mutants described in the accompanying paper (20), these mutants have allowed us to define functional domains in the OxyR protein.

C-199 as a possible redox center. Using site-directed mutagenesis, we found that only one (C-199) of six cysteine residues was critical for the induction of defense genes by OxyR. The C199S mutant was unable to activate the antioxidant genes and gave rise to an extended footprint characteristic of reduced OxyR. Since oxidation is necessary for OxyR to activate transcription in vitro, we suggest that C-199 is the redox center of the protein and that the C199S protein is locked in the reduced conformation. Cysteines are known to be redox-reactive amino acids and can form inter- or intramolecular disulfide bridges. However, an intramolecular disulfide bridge is unlikely to be the redox-active center of OxyR, since only C-199 proved to be critical, and the mutant in which all other cysteines were mutated to serine was still active in vivo. We did not observe any differences between the wild-type and mutant C199S proteins on nonreducing gels which permit the resolution of disulfide-linked oligomers (data not shown), suggesting that intermolecular disulfide bridges do not constitute the redox-active center. Redox-active metals are also coordinated by cysteine residues. The SoxR protein, a transcriptional activator in the bacterial response to superoxide, carries a nonheme iron which is likely to be the redox-reactive center of the SoxR protein (17). However, we do not think that a metal coordinated through C-199 is the redox-active center of OxyR, since the addition of chelators did not affect transcriptional activation of OxyR in vitro (35). Cysteines have been shown to be reversibly oxidized to sulfenic (SO^-) or sulfinic (SO_2^-) acid and irreversibly oxidized to sulfonic (SO_3^-) acid. Both a streptococcal NADH peroxidase and an oxidation product of the protease papain have been reported to have a cysteine-sulfenic acid (22, 27). Since OxyR is reversibly oxidized and only a single cysteine is critical for OxyR activity, we suggest that C-199 is oxidized to a sulfenic acid. The mutants described

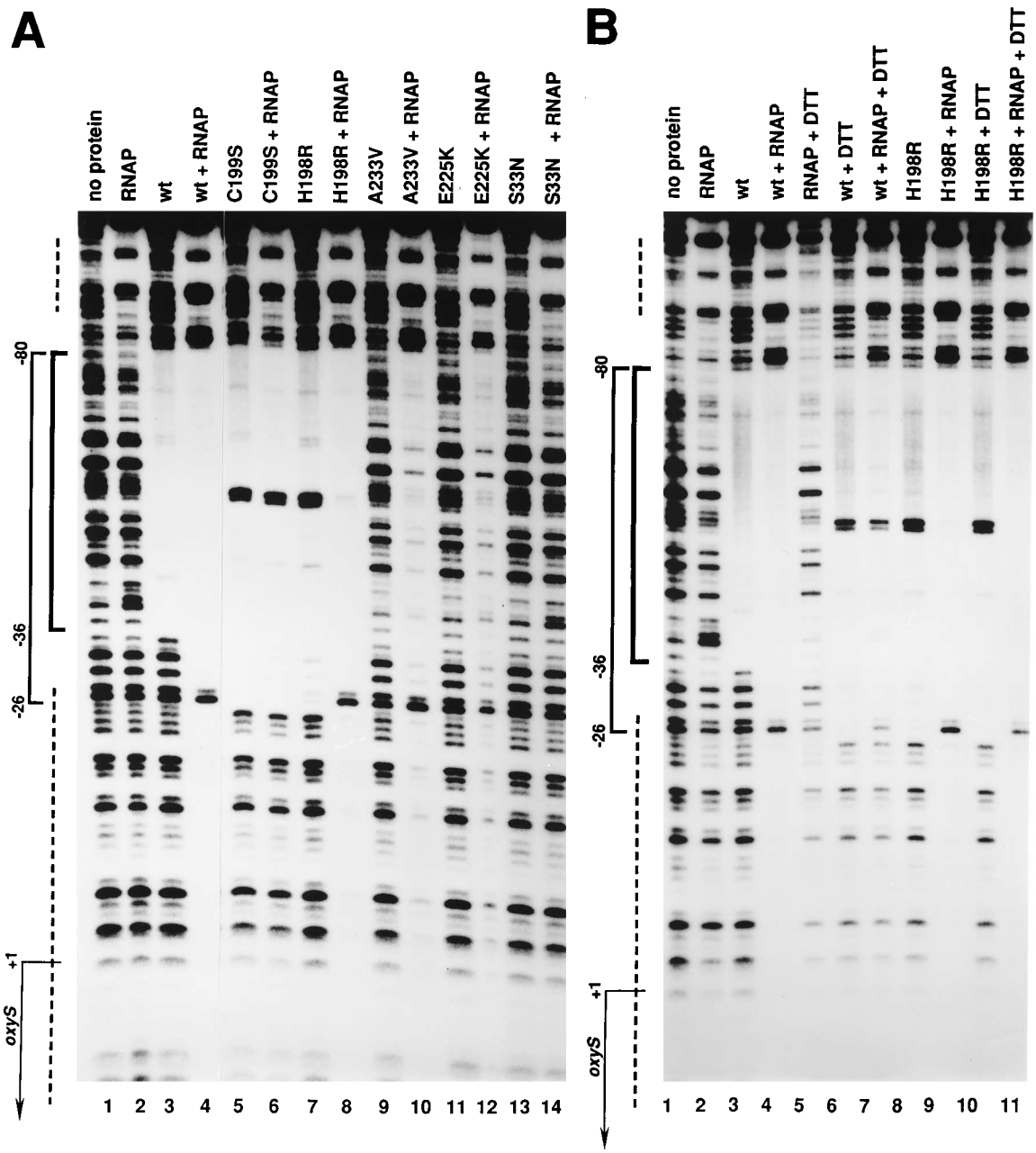

FIG. 7. DNase I footprints of RNA polymerase (RNAP) together with wild-type (wt) and mutant OxyR proteins on the bottom strand of the *oxyS* promoter fragment. The 200-bp *Eco*RI-*Hin*dIII fragment of pGSO43 (41) labeled at the *Eco*RI site was incubated with purified RNA polymerase and the purified wild-type and OxyR mutant proteins as indicated. (A) Footprinting assays carried out in the absence of DTT; (B) RNA polymerase binding assayed in the absence or presence of 200 mM DTT. The heavy brackets denote the oxidized footprint of OxyR, the light brackets denote the reduced extended footprint of OxyR, and the dotted lines indicate the sequences protected by RNA polymerase binding. The positions of the footprints are labeled with respect to the start of the *oxyS* transcript. The upper protection by RNA polymerase does not correspond to either the *oxyS* or *oxyR* promoter and is assumed to be due to nonspecific binding to the end of the fragment.

here will be useful for future biochemical studies of the modifications of the cysteine residues in OxyR.

Constitutively active mutants define inducer-responsive and activation domains. Using a random mutagenesis approach, we isolated eight mutants which constitutively activate transcription of their target genes during normal growth and show increased resistance to oxidants. The corresponding mutations can be grouped and map to domains that may be functionally conserved among the LysR family members. Two of the amino acid substitutions (T100I and H114Y) map to a region (residues 98 to 150) that is somewhat conserved among the LysR proteins and that has been defined as a coinducer recognition/response domain by mutations in other LysR family members (29). Four of the mutations (H198Y, H198R, R201C, and C208Y) map within or near a second coinducer recognition/response domain (residues 196 to 206) of the LysR family (29). These four mutations are located close to the proposed redox-active C-199 residue, consistent with the conclusion that this region is the hydrogen peroxide-responsive domain. Finally, three of the mutations (A233V, A233T, and G253K) are lo-

cated near a carboxy-terminal region (residues 227 to 253) that shows some homology between the LysR proteins. Given the partial conservation of residues 227 to 253 and residues 98 to 150, these regions might be involved in touching RNA polymerase. Experiments described in the accompanying paper also indicate that the C-terminal domain is involved in oligomerization of OxyR (20).

It is not clear whether the critical C-199 residue is still modified by oxidation in the constitutive mutants. However, since the T100I and H114Y mutants still showed a two- to threefold increase of activation upon oxidation, these mutants should still be redox reactive. Three of the mutations causing a constitutively active phenotype affected histidine residues (H114Y, H198Y, and H198R). The crystal structures of the streptococcal NADH peroxidase and the oxidized papain (the two proteins found to have a sulfenic acid) showed that histidine residues form hydrogen bonds with the redox-active cysteine (19, 32). If a sulfenic acid proves to be the redox center of OxyR, the H-114 and H-198 residues may act to form hydrogen bonds with the sulfenic acid. We suggest that the amino acids of the constitutive mutants may more effectively stabilize the negatively charged residue and allow the oxidation of C-199 during normal growth. Structural studies of OxyR should help to elucidate whether the regulator carries a sulfenic acid stabilized by histidine residues and give additional insights into the domains of OxyR and other LysR family proteins.

Cooperative binding with RNA polymerase. The overall activities of the constitutive mutants varied from 3 to 400% of the wild-type activity in vivo. It was not possible to correlate the variations in activity with differences in DNA binding. In fact, we were surprised to find that although one constitutive mutant gave rise to a short oxidized footprint, several constitutive mutants showed reduced extended footprints, and some of the mutants showed no footprint at all. The different footprint phenotypes were specific to different regions, since both T100I and H114Y had decreased binding and all of the mutants with substitutions near C-199, including the C208S mutant generated by site-directed mutagenesis, gave extended footprints. The ability to activate transcription, however, did correlate with the abilities of the purified proteins to bind cooperatively with RNA polymerase. The uninducible C199S mutant did not induce transcription and was unable to stimulate RNA polymerase binding. In contrast, the constitutive H198R mutant, which showed the same extended reduced footprint seen for C199S in the absence of RNA polymerase, induced polymerase binding even in the presence of 200 mM DTT. Another constitutive mutant, A233V, showed significantly reduced binding to DNA alone but also induced polymerase binding.

RNA polymerase binding to the promoter can be a key step in the activation by transcription factors. At some bacterial promoters, RNA polymerase is unable to bind in the absence of a transcriptional regulator, preventing the titration to these promoters when the corresponding genes need not be activated. For example, RNA polymerase is unable to bind to the promoters of the *pho* (phosphate) genes in the absence of the PhoB activator (23). We propose that the oxidation of the wild-type OxyR protein exposes a domain which allows OxyR to recruit RNA polymerase and that the constitutive phenotype of our mutants may be due to exposure of this domain under both oxidizing and reducing conditions.

Our results also suggest that while OxyR induces RNA polymerase binding, RNA polymerase has an effect on OxyR. Both A233V and E225K did not shown DNase I footprints when incubated with the *oxyS* promoter alone but did bind in the presence of RNA polymerase. We propose that the contact with polymerase may also cause the H198R mutant to adopt the oxidized configuration, since the DNase I-hypersensitive site in the center of the reduced H198R footprint disappears upon the interaction with RNA polymerase. An important direction for further studies will be to elucidate this reciprocal interaction between OxyR and RNA polymerase.

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REFERENCES

1. **Adhya, S.** Unpublished data.
2. **Adhya, S., and S. Garges.** 1990. Positive control. J. Biol. Chem. **265:**10797–10800.
3. **Altuvia, S., M. Almirón, G. Huisman, R. Kolter, and G. Storz.** 1994. The *dps* promoter is activated by OxyR during growth and by IHF and σ^s during stationary phase. Mol. Microbiol. **13:**265–272.
4. **Altuvia, S., and G. Storz.** Unpublished data.
5. **Bolivar, F., and K. Backman.** 1979. Plasmids of *Escherichia coli* as cloning vectors. Methods Enzymol. **68:**245–267.
6. **Bölker, M., and R. Kahmann.** 1989. The *Escherichia coli* regulatory protein OxyR discriminates between methylated and unmethylated states of the phage Mu *mom* promoter. EMBO J. **8:**2403–2410.
7. **Brosius, J., and A. Holy.** 1984. Regulation of ribosomal RNA promoters with a synthetic *lac* operator. Proc. Natl. Acad. Sci. USA **81:**6929–6933.
8. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. BioTechniques **5:**376.
9. **Burn, J. E., W. D. Hamilton, J. C. Wootton, and A. W. B. Johnston.** 1989. Single and multiple mutations affecting properties of the regulatory gene *nodD* of *Rhizobium*. Mol. Microbiol. **3:**1567–1577.
10. **Calos, M. P.** 1978. DNA sequence for a low-level promoter of the *lac* repressor gene and an "up" promoter mutation. Nature (London) **274:**762–765.
11. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
12. **Chang, M., and I. P. Crawford.** 1990. The roles of indoleglycerol phosphate and the TrpI protein in the expression of *trpBA* from *Pseudomonas aeruginosa*. Nucleic Acids Res. **18:**979–988.
13. **Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames.** 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. Cell **41:**753–762.
14. **Christman, M. F., G. Storz, and B. N. Ames.** 1989. OxyR, a positive regulator of hydrogen peroxide-inducible genes in *Escherichia coli* and *Salmonella typhimurium*, is homologous to a family of bacterial regulatory proteins. Proc. Natl. Acad. Sci. USA **86:**3484–3488.
15. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics: a manual for genetic engineering. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Fisher, R. F., and S. R. Long.** 1992. *Rhizobium*-plant signal exchange. Nature (London) **357:**655–660.
17. **Hidalgo, E., and B. Demple.** 1994. An iron-sulfur center essential for transcriptional activation by the redox-sensing SoxR protein. EMBO J. **13:**138–146.
18. **Huang, J., and M. A. Schell.** 1991. *In vivo* interactions of the NahR transcriptional activator with its target sequences: inducer-mediated changes resulting in transcription activation. J. Biol. Chem. **266:**10830–10838.
19. **Kamphuis, I. G., K. H. Kalk, M. B. A. Swarte, and J. Drenth.** 1984. Structure of papain refined at 1.65 Å resolution. J. Mol. Biol. **179:**233–256.
20. **Kullik, I., J. Stevens, M. B. Toledano, and G. Storz.** 1995. Mutational analysis of the redox-sensitive transcriptional regulator OxyR: regions important for DNA binding and multimerization. J. Bacteriol. **177:**1285–1291.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
22. **Lin, W. S., D. A. Armstrong, and G. M. Gaucher.** 1975. Formation and repair of papain sulfenic acid. Can. J. Biochem. **53:**298–307.
23. **Makino, K., M. Amemura, S.-K. Kim, A. Nakata, and H. Shinagawa.** 1993. Role of the σ^{70} subunit of RNA polymerase in transcriptional activation by activator protein PhoB in *Escherichia coli*. Genes Dev. **7:**149–160.
24. **McIver, J., M. A. Djordjevic, J. J. Weinman, G. L. Bender, and B. G. Rolfe.** 1989. Extension of host range of *Rhizobium leguminosarum* bv. *trifolii* caused by point mutations in *nodD* that result in alterations in regulatory function and recognition of inducer molecules. Mol. Plant-Microbe Interact. **2:**97–106.

25. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Ostrowski, J., and N. M. Kredich.** 1989. Molecular characterization of the *cysJIH* promoters of *Salmonella typhimurium* and *Escherichia coli*: regulation by *cysB* protein and *N*-acetyl-L-serine. J. Bacteriol. **171:**130–140.
27. **Poole, L. B., and A. Claiborne.** 1989. The non-flavin redox center of the streptococcal NADH peroxidase. J. Biol. Chem. **264:**12330–12338.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Schell, M. A.** 1993. Molecular biology of the LysR family of transcriptional regulators. Annu. Rev. Microbiol. **47:**597–626.
30. **Schell, M. A., P. H. Brown, and S. Raju.** 1990. Use of saturation mutagenesis to localize probable functional domains in the NahR protein, a LysR-type transcription activator. J. Biol. Chem. **265:**3844–3850.
31. **Spaink, H. P., C. A. Wijffelman, R. J. H. Okker, and B. E. J. Lugtenberg.** 1989. Localization of functional regions of the *Rhizobium nodD* products using hybrid *nodD* genes. Plant Mol. Biol. **12:**59–73.
32. **Stehle, T., S. A. Ahmed, A. Claiborne, and G. E. Schulz.** 1991. Structure of NADH peroxidase from *Streptococcus faecalis* 10C1 refined at 2.16 Å resolution. J. Mol. Biol. **221:**1325–1344.
33. **Storz, G., and S. Altuvia.** 1994. OxyR regulon. Methods Enzymol. **234:**217–223.
34. **Storz, G., M. F. Christman, H. Sies, and B. N. Ames.** 1987. Spontaneous mutagenesis and oxidative damage to DNA in *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **84:**8917–8921.
35. **Storz, G., L. A. Tartaglia, and B. N. Ames.** 1990. Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. Science **248:** 189–194.
36. **Tao, K., N. Fujita, and A. Ishihama.** 1993. Involvement of the RNA polymerase α subunit C-terminal region in co-operative interaction and transcriptional activation with OxyR protein. Mol. Microbiol. **7:**859–864.
37. **Tao, K., K. Makino, S. Yonei, A. Nakata, and H. Shinagawa.** 1989. Molecular cloning and nucleotide sequencing of *oxyR*, the positive regulatory gene of a regulon for an adaptive response to oxidative stress in *Escherichia coli*: homologies between OxyR protein and a family of bacterial activator proteins. Mol. Gen. Genet. **218:**371–376.
38. **Tao, K., K. Makino, S. Yonei, A. Nakata, and H. Shinagawa.** 1991. Purification and characterization of the *Escherichia coli* OxyR protein, the positive regulator for a hydrogen peroxide-inducible regulon. J. Biochem. **109:**262–266.
39. **Tartaglia, L. A., G. Storz, and B. N. Ames.** 1989. Identification and molecular analysis of *oxyR*-regulated promoters important for the bacterial adaptation to oxidative stress. J. Mol. Biol. **210:**709–719.
40. **Thomas, M. S., and R. E. Glass.** 1991. *Escherichia coli rpoA* mutation which impairs transcription of positively regulated systems. Mol. Microbiol. **5:**2719–2725.
41. **Toledano, M. B., I. Kullik, F. Trinh, P. T. Baird, T. D. Schneider, and G. Storz.** 1994. Redox-dependent shift of OxyR-DNA contacts along an extended DNA binding site: a mechanism for differential promoter selection. Cell **78:**897–909.
42. **Wang, L., J. D. Helmann, and S. C. Winans.** 1992. The *A. tumefaciens* transcriptional activator OccR causes a bend at a target promoter, which is partially relaxed by a plant tumor metabolite. Cell **69:**659–667.
43. **Warne, S. R., J. M. Varley, G. J. Boulnois, and M. G. Norton.** 1990. Identification and characterization of a gene that controls colony morphology and auto-aggregation in *Escherichia coli* K12. J. Gen. Microbiol. **136:**455–462.