Mutational Analysis To Define an Activating Region on the Redox-Sensitive Transcriptional Regulator OxyR[⊽]

Xunde Wang,† Partha Mukhopadhyay,‡ Matthew J. Wood,§ F. Wayne Outten, Jason A. Opdyke, and Gisela Storz*

Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland

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The OxyR transcription factor is a key regulator of the *Escherichia coli* response to oxidative stress. Previous studies showed that OxyR binding to a target promoter enhances RNA polymerase binding and vice versa, suggesting a direct interaction between OxyR and RNA polymerase. To identify the region of OxyR that might contact RNA polymerase, we carried out alanine scanning and random mutagenesis of *oxyR*. The combination of these approaches led to the identification of several mutants defective in the activation of an OxyR target gene. A subset of the mutations map to the DNA-binding domain, other mutations appear to affect dimerization of the regulatory domain, while another group is suggested to affect disulfide bond formation. The two mutations, D142A and R273H, giving the most dramatic phenotype are located in a patch on the surface of the oxidized OxyR protein and possibly define an activating region on OxyR.

The OxyR transcription factor was originally identified as a regulator of the Salmonella enterica serovar Typhimurium and Escherichia coli responses to hydrogen peroxide but has since been discovered in many bacterial species (reviewed in references 13 and 21). The protein is both the sensor and the transducer of peroxide stress. During normal growth, OxyR is reduced and acts as a repressor of a subset of genes, including negative autoregulation of its own expression. Upon exposure to elevated levels of hydrogen peroxide, OxyR is oxidized and activates the expression of a regulon of genes encoding defense activities. In previous studies, we showed that OxyR is activated by reversible disulfide bond formation between cysteine residues 199 and 208 (1, 9, 26) and that the reduced and oxidized forms of the tetrameric transcription factor make different contacts along the promoter DNA (25). The solutions of the crystal structure of the regulatory domain (residues 80 to 305) in the reduced and oxidized conformation showed that the two redox-active cysteines are approximately 17Å apart in the reduced structure and that disulfide bond formation in the oxidized form results in a significant structural change in the regulatory domain (3). The different orientations of the monomers relative to each other in the reduced and oxidized conformations can explain the different DNA-binding footprints observed for the two forms of OxyR.

OxyR is a member of the LysR family of transcriptional

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regulators. This family comprises the most abundant class of transcriptional regulators in bacterial cells (12). Most of the LysR-type regulators are between 30 and 35 kDa in molecular size and form homodimers or homotetramers. The proteins have a very conserved amino-terminal domain containing a helix-turn-helix DNA-binding motif. Unlike OxyR, most of the LysR family members are activated by binding to effector molecules. However, for all of the LysR-type transcriptional regulators, the carboxy-terminal part of the protein constitutes the inducer-binding or regulatory domain.

OxyR, like most of the LysR family members, binds overlapping or adjacent to the promoter to repress or activate transcription (reviewed in reference 16). Footprinting experiments showed oxidized OxyR increases RNA polymerase binding to the OxyR-dependent promoters, suggesting that OxyR activates transcription by recruiting RNA polymerase (8). This interaction may be due to direct contacts between the α subunit of RNA polymerase (encoded by rpoA) and OxyR, since strains expressing α mutants lacking the carboxy-terminal domain (α -CTD) are unresponsive to OxyR activation (22). A screen for rpoA mutations that resulted in decreased OxyR activation of the *katG* target gene led to the isolation of 11 α mutants with substitutions for amino acids 265, 268, 269, 293, 294, 298, 299, 300, and 307 in the α -CTD (23). However, it has not been established whether OxyR makes direct contacts with this domain of RNA polymerase. Since all of the mutations map to the α DNA-binding domain, the reduced activation observed may solely be due to reduced RNA polymerase binding to the katG, ahpC, and oxyS promoters rather than an OxyR contact site on the α -CTD. To gain insight into possible contacts between OxyR and RNA polymerase, we carried out alanine scanning and random mutagenesis of oxyR and screened for mutants unable to activate transcription.

MATERIALS AND METHODS

Plasmids and bacterial strains. Derivatives of pAQ5 (20) were used for all phenotypic assays. For the overexpression and purification of mutant OxyR

^{*} Corresponding author. Mailing address: NIH, Building 18T, Room 101, 18 Library Dr., MSC 5430, Bethesda, MD 20892-5430. Phone: (301) 402-0968. Fax: (301) 402-0078. E-mail: storz@helix.nih .gov.

[†] Present address: Vascular Medicine Branch, National Heart, Lung, and Blood Institute, Bethesda, MD 20892.

[‡] Present address: Laboratory of Physiologic Studies, National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD 20852.

[§] Present address: Department of Environmental Toxicology, University of California, Davis, CA 95616.

^{||} Present address: Department of Chemistry and Biochemistry, The University of South Carolina, Columbia, SC 29208.

TABLE 1. E. coli strains used for this study

Strain	Relevant genotype	Source or reference		
DH5a		BRL		
XL1-Blue		Stratagene		
XL1-Red	<i>mutD5 mutS mutT</i> Tn10(tet)	Stratagene		
TA4484	$oxyR\Delta3/pMC7$	24		
N9716	GC4468 $\Delta oxyR$::Sp	W. Gillette		
FÅ369	$\Delta ahpCF$::Kan	1		
GSO5	$\Delta oxyR$::Kan $\Phi(oxyS-galK)$	8		
GSO130	MC4100 $\Phi(oxyS-lacZ)$	25		
GSO131 (WX16)	MC4100 $\Phi(oxyS-lacZ) \Delta oxyR::Sp$	This study		
GSO132 (WX21)	MC4100 $\Phi(oxyS-lacZ) \Delta oxyR::Sp$ $\Delta ahpCF::Kan$	This study		
GSO133	MC4100 $\Phi(oxyR-lacZ)$ $\Delta oxyR::Kan$	25		

proteins, the 0.8-kb RsrI/HindII *axyR* fragment from the pAQ5 derivatives was used to replace the same fragment in pGSO69 (8). The bacterial strains used in the present study are listed in Table 1. DH5 α and XL1-Blue were routinely used for plasmid preparation. GSO5 (8) was used for oxidant sensitivity test and for primer extension assays, TA4484 (24) was used for the overexpression and purification of mutant OxyR proteins, and GSO133 (25) was used for the *axyR*-*lacZ* fusion assays. The *axyR*::Sp deletion was moved into GSO130 (25) by P1 transduction from N9716 (kindly provided by W. Gillette) to generate GSO131 (WX16). The *ahpCF*::Kan deletion similarly was introduced into GSO131 by P1 transduction from FÅ369 (1) to construct GSO132 (WX21). P1 transductions were carried out as described previously (18).

Growth conditions. Strains were routinely grown at 37°C in Luria-Bertani (LB) medium (11). Ampicillin (50 to 100 μ g/ml), chloramphenicol (25 μ g/ml), kanamycin (Kan; 25 μ g/ml), streptomycin (Sp; 25 μ g/ml), or 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal; 40 μ g/ml) was added where appropriate. Mutants were identified on MacConkey or tetrazolium medium containing 1% lactose (11, 18).

Mutagenesis. The desired alanine substitutions were generated by using the QuikChange site-directed mutagenesis kit (Stratagene) and oligonucleotides carrying the appropriate codon substitutions. Two approaches were used to create the random mutations in the *axyR* gene carried on pAQ5. First, pAQ5 was transformed into a mutator strain (XL1-Red). The plasmid isolated from about 500 transformants grown in LB medium with chloramphenicol for ~24 h was then used to transform GSO132. As a second approach, chemical mutagenesis of pAQ5 was carried out as described previously (8). Briefly, 20 μ l of pAQ5 plasmid DNA (5 μ g) was mixed with 80 μ l of 0.5 M potassium phosphate buffer (pH 6.0) containing 5 mM EDTA and 100 μ l of 1 M hydroxylamine. The mixture was incubated at 65°C for 60 min. The DNA was dialyzed extensively with Tris-Cl-EDTA buffer and then used to transform XL1-Blue. The XL1-Blue transformants were collected, and plasmid DNA was again isolated and used to transform GSO132. For each mutant, the sequence of the entire *axyR* gene was confirmed by sequencing.

Zone-of-inhibition assays. Aliquots (0.1 ml) of overnight cultures were mixed with 2.5 ml of top agar and plated on LB medium containing the appropriate selection. Disks impregnated with 10 μ l of either 10% H₂O₂ or 4% cumene hydroperoxide were placed on the plates. The zones of inhibition surrounding the disks were measured after overnight incubation.

Primer extension assays. Cultures were grown to an optical density at 600 nm of 0.3 to 0.5 and split; half was left untreated, and the other half was exposed to 0.2 mM hydrogen peroxide. The cultures were shaken for 5 min, and the cells were collected. The total RNA was extracted by using TRIzol reagent (BRL). Primer extension assays were carried out with 5 μ g of total RNA and an oligonucleotide (5'-GCAAAAGTTCACGTTGG) complementary to the *axyS* gene that was labeled with T4 polynucleotide kinase. The probe was annealed to the RNA, and a 60-min extension reaction was carried out at 42°C using reverse transcriptase (Life Sciences Inc.). The products were analyzed by using an 8% sequencing gel.

Protein expression and purification. The wild-type and mutant OxyR proteins were overexpressed and purified as described previously (8). Briefly, TA4484 carrying the pGSO69 derivatives was grown to an optical density at 600 nm of 0.5, and expression was induced by 0.5 mM IPTG (isopropyl- β -D-thiogalactopyranoside) for 2 h. Cells were harvested and lysed by three passages through a French

press. Cell debris was removed by centrifugation, and the OxyR protein in the supernatant was purified by passage over heparin-Sepharose and Mono-S columns (Pharmacia).

DNase I footprinting assays. DNase I footprinting assays were carried out as described previously (24). An end-labeled DNA fragment was incubated with 50 to 200 ng of purified protein in 25 μ l of 0.5× TM buffer. The binding reaction mixtures were then treated with DNase I for 2 min, extracted with phenol-chloroform, and examined on 8% sequencing gels.

Immunoblotting. α -OxyR antiserum was generated by immunizing rabbits with purified OxyR protein (Covance). Total proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% acrylamide and transferred to a nitrocellulose filter by electroblotting. The filter was probed with a 1:10,000 dilution of antiserum. Bound antibody was visualized by rabbit antiserum using the enhanced chemiluminescence Western blotting system from Amersham.

 β -Galactosidase assays. β -Galactosidase assays were carried out according to the method of Miller (11).

RESULTS AND DISCUSSION

Alanine-scan screen for activation-defective mutants. The DNA-binding domain of LysR type factors is highly conserved and located at the amino termini of these proteins (reviewed in reference 16). Based on the assumption that regions important for the interaction with RNA polymerase also may be somewhat conserved among LysR family proteins, we aligned several E. coli members of this family of transcriptional regulators (Fig. 1). We noticed two patches of conservation in addition to the DNA-binding domain. One region spans residues 135 to 145, and another region spans residues 231 to 241, encompassing the position of the A233V mutation found for the oxyR2 constitutive mutant (4). To determine whether any of these residues are important for transcriptional activation by OxyR, we substituted alanine for each of the amino acids. The mutant derivatives carried on the pACYC184 plasmid were then introduced into the oxyR deletion strain GSO5, and the strains were examined for their sensitivity to hydrogen peroxide and cumene hydroperoxide (Table 2). These assays revealed that two substitutions, D142A and T238A, resulted in significantly increased sensitivity to both oxidants. All other mutants showed wild-type or only slightly increased sensitivity to the peroxides. The expression of oxyS, an OxyR target gene, also was examined by primer extension assays in these mutant strains (Fig. 2). The results of the primer extension assay were consistent with those of the oxidant inhibition test. The D142A and T238A mutants showed significantly decreased induction of the OxyR target gene, indicating that the two mutants cannot activate transcription in response to hydrogen peroxide treatment. The L239A and V231A mutants also showed reduced induction of the OxyS RNA in the primer extension assay but were not hypersensitive to the oxidants. For the L239A mutant, the wild-type resistance most likely is due to the partially constitutive nature of this mutant; some OxyS expression was observed even in the absence of hydrogen peroxide treatment.

The T238A mutant is locked in a reduced conformation. Examination of the structure of the OxyR regulatory domain, solved during the course of these studies (3), showed that the T238 residue is buried in the core of the region containing the redox-active C199 and C208 residues involved in disulfide bond formation. This proximity of the T238 residue to the redox-active cysteines raised the possibility that the activation defect in the T238A mutant might be due to defective disulfide

		R4C L6P	Y8H					A22V			D301		S33N										
OxyR	M	NIRD	EYL	ALA	EHF	H-	FRF	AA	DSC	HV	SQI	TL	SG	QI	RK	LE	DE	LG	VM	L	ER	TS	-
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MetR	MI	EVKHL	KTLÇ	DALR	NCO	- s	LAA	AA	ATL	HQ	TQS	AL	SH	QF:	SD	LE	QR	LG	FR	LF	VR	КS	- 1
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OxyR CysB IlvY MedcA KapR OxyR CysB IlvY	EDHPWANI PDHPLAGI PALPCPVI KSRTCTG EEHHLSSI NMVAAGS TYVRLGL SMVALGC	R EC R EA RNQVS K TR F - ST 1 W822 UTLL GUGVI GVGVI GVALL	VPMA ITIE VEKP ITPE LES- VPLE 80 PALA PALA PEVV	VP- VP- VD- VLEN	GEK QYP SET NEQ DEY 1	LLI LV FII LL FV 90	MLE TYT MAD LPQ TMP RDG DPD RNR		A6613-CLLGYVRVLCLGYVRVLCLGYVRVLCLGYVRVLCLGYVRVLCLGYVRVLCLGYVRVLCLGYVRVLCLGYVRVLCLGYVRVLCLGYVRVLCLGYVRVLCLGYVRVLCLGYVRVLCLGYVRVLCLGYVRVLCLGYVRVLCLGYVRVLCLGYVRVLCGYVRVLCGYVRVLCGYVRVLCGYVRVLGVVRVLGYVRVLgyvrvrvrvcgvrvrvcqvrvrvcqvrvrvcqvrvrvcqvrvcq	RD(RSI RR: LDY WDI 000 CII RS:	QAM ELD IEL VWR ELL FLQ KPHFK	GF TA WF HF TT RV 	CFI FNI RRI LQI CQC RT: ST: FEI	EAG RAG NKI PAG 2VG 210 IGI IGI		CEI NPA SII SP HEZ3H FRI AQI	OTI RIV SLI VV: PG: RS ¹	STF	RATATVD KTV 22 LRR -K	TTDGNSE SSYL	LEDADETLVQ YEYHE	TL VI AM LL TI V 	RKVIYL.
OxyR CysB IlvY MedcA KapR OxyR IlvY MedcA	EDHPWANI PDHPLAGI PALPCPVI KSRTCTG EEHHLSSI NMVAAGS TYVRLGL SMVALGC QMVAARM	120 REC KKA RNQVS KTR FST FST FST FST CUSUL CUSUL CUALL CUALL CUALL CUALL	VPMA ITIE VEKF LES- VPLE 80 PALA PALA PEVV	VP - VVP - VVD - VVE -	GEK QYF SET DEY 1 -PH F	LLI LV FII LL FV 90 RK	MLETYT MADDY LPQP RDPD RNR RQG		A6619-CLGGYVR FTRAGYUL AGUULAN AGUULAN AGUULAN AGUULAN AGUULAN	RD(RSI RR: LD' YSI WDI 00 CII AHI RSI TL	QAM ELD IEL VWR ELL FLQ KPEFLQ C	GF TA WF HF TT RV 	CFI FNI RRI LQI CQC ST SFEI SRI	EAG RAG RAG 200 210 IGI TK: LGV	E AI E SI E SI E F S E F	CELEVILLE CONTRACTOR C	PG C- C- C- C- C- C- C- C- C- C- C- C- C-	16 S6124-FFASVR	RAATVORVEV22 LRRKQ	TTDGNSE SSYLVA	LEDELTLVQ YMHEE	TL VI AM LL TI VI AM LL TI VI AM LL TI VI AM	RKVIYL ·
OxyR CysB IlvY MetcA KapR OCysB IlvY MetcA XapR	EDHPWANI PDHPLAGI PALPCPVI KSRTCTG EEHHLSSI NMVAAGS TYVRLGL SMVALGC QMVAARM NLVLNAD AMVSMGI	RECA RECA RNQVS KTR FTT FST FST V& V& V& V& V& V& V& V& V& V& V& V& V&	VPMA ITIE VEKF VEKF LES- VPLE 80 PPLE 80 PALA PEWV PALA	ADEA EEA DWS CDEA CALR CALR CALR CALR CALR CALR CALR CAL	GEK QYP SET NEQ DEY 1	LLI LV FII LL WV FV 90 RK VA EV FG MN	MLET MYTADP LIPQ MUTADP CD RRQQ QD RRQQQ WPG	UVV UVV UVV UVV UVV UVV UVV UVV	· A661-CTGRRYD2 PDEKPP	RD(RSI RR: LD YSI WDI 000 CII RSI TL VE: -LI	QAM ELD IELR FLQ FLQ FLQ FLQ FLQ FLQ FLQ	GF TA WF TT RV 	CFI RRI LQI CQ(RT: STI SRI AQI	EAC RAC NKI PAC 210 IGI IKI LGV LVI YAI	LVS LVS LVS LVS LVS LVS LVS LVS LVS LVS	CEPH CEPH SEEN HEZZAR H	PG: Construction	- Selicity - Stranger	RAATVDTVE22 LRRKQK	TTDG-SE SSRPKOV	LEDELVQ · YMHESP	TL VI ALL TI TV OLF PLF VL	RKVIYL ·
OxyR CysB Ilvr IdcA XapR OxyR Ilvy MetR XapR	EDHPWANN PDHPLAGI KSRTCTG EEHHLSSI NMVAAGS TYVRLGL SMVALGC QMVAARM NLVLNAD AMVSMGI .230	R EC K KA RNQVS K - TTT F - ST F - ST SITLL SVGVI GVALL GIAAL FLTVI GIAAL FLTVI GIAAL SITLLI	VPMA ITIE VEKF ITPE LES- VPL 80 PALA ASMA PEWV PCDW ADSY 40	ADEA BELA DUSS CDEA COMA CALR AUP- VUE- VUE- VUE- VUE- VUE- VUE- VUE- VUE	GEKF QYF SET NEQ DEY 1 -PH SPH FF FF	LLI LV FII LL FV 90 RK PV FV FG MN .2	MLET TYT MAD LPQ TMP CTMP CTMP CTMP CTMP CTMP CTMP CTMP	UVV UVV UVV UVV UVV UVV UVV UVV UVV UVV	AGGLJ-LLGGXGYD2 AGGLJ-CLGGXGYD2 AGGLJ-CLGXGYD2 AGGLJ-CCGXGYD2 AGGLJ-CCGXGYD2 AGGLJ-CCGXGYD2 AGGL	RD(RRSI RRSI VSI WDD 00 CII RSI TLO VE: -L1 .2	QLUERF	GFF TA WFF TT RV PR SHP LW PV IP	CFI FRI LQI CQC RT: SRI SRI AQI	EAC RAC NKI PAC RNC 210 IGI IK: LGV LYA YAY	AI GLU SI SI SI SI SI SI SI SI SI SI SI SI SI	CELEVICE CONTRACTOR CO	PG: Control Control Co	ST-QR	RAATVDTVEV2 LRRKQL-Q.	TTDGNSE SSYLVA 28	LEDELTVQ. YMHEESP.	TL VI AM LL TI TV QLF PLF VAM	RKVIYL ·
OxyR CysB Ilvr IdcA KapR OxyR BIlvY MetR XapR XapR	EDHPWANI PDHPLAGI KSRTCTG EEHHLSSI NMVAAGS TYVRLGL SMVALGC QMVAARM NLVLNAD AMVSMGI .230 EAIRARM	R ECK R KA RNQVS K TT F - ST V V E V C V S S V S S S V S S S S S S S S S S S S S	VPMA ITIE VEKF ITPE 80 PALA PALA SMA PEVW PCDN ADSY 40 KVLK	ADEA EELA DWS CDA CALK CALR CALR CALR CALR CALR CALR CALR CALR	GEKF QYP TVF SET NEQ DEY 1	LLI LV FII LL WV FV 90 RK VA FV FV 2 - - - - - - -	MLET TYT MAD LPAP TMP RD DPD RQ QQ WPG 50.	UVV UVV UVV UVV UVV UVV UVV UVV	- A6613-CLIGTOR RAGIN	RD(RRS) RRC YSI WDD 00 CII RRS TLC VE: -LI .2	AMD CELLQ CELL	GFTA WFF TTT RV PR SHPV IP	CFII FRI LQI CQ(STT SFEI SRI AQI	EAG RAG NKI PAG 210 IGI IGI IK: LGV LYI LYI	AI ISI ISI ISI ISI ISI ISI ISI I	CPH SSIT VPS SSIT VPS SSIT VS SSIT SSIT	PG: C- C- C- C- C- C- C-	16 SOLT ST - QR	C RAATVDTVE22 LRRKQKI-Q.	TTDG-SEVLVAQ28	LEDELTVQ YMHEESP.	TL VI AM LLL TI TV QLF PL AF	RKVIYL · AIIIIVV ·
OxyR CysB Ilvr IdcA KapR OxyR BIlvY MetcA KapR XapR OxyR BCXSB	EDHPWANI PDHPLAGI KSRTCTG EEHHLSSI NMVAAGS TYVRLGL SMVALGC QMVAARC NLVLNAD AMVSMGI .230 EAIRARMI QRFAPHL	R ECK K KA RNQVS K TT F - ST F - ST V& EITLLI GVGVI GVALL GI	VPMA ITIE VEKF ITPE 80 PALA PASMA PEVW PCDM ADSY 40 KVLK	ADEA EELA DWS CDWS CDWS CDWS CDWS CDWS CDWS CDWS	GEKF QYF SET SET DEY 1	LLI LV FII LL WV FV 90 RK VA FV FG MN .2 EI	MLET MADD IYAD IYAD IYAD MADD IYAD NADD IYAD NADD IYAD NADD IYAD NADD IYAD NADD IYAD NADD IYAD NADD IYAD IYAD IYAD IYAD IYAD IYAD IYAD I	SVVS SVVS SVVS SVVS SVVS SVVS SVVS SVV	· A66L9-CLIGTRRY SCART LVLEKP CRUTTRRK	RD(RSI RR: LD YS) WD 00 CII RRS TL VE: -LI .2 	AMD AMD ALL LUVELL V	GF TA WF TT RV 	CFII FNI LQI CQQ RT: STE SRI AQD	EAG RAG PAN 2210 IGI IGI IGI LGY YAY	LVS LVS LVS LVS LVS LVS LVS LVS LVS LVS	CIPPISIE HELLS	PG: C- CC- CC-	I SOLA-FFASVIN ST-QR	RAATVDKTV22 LLRKQKQ	TSDGGN- GGN- DNE SSRLVAV28	LEDELTUVPQ · · · · · · · · · · · · · · · · · · ·	TL VI AM LL TI TV ODF PL AF	RKVIYL ·
OxyR CysB IlvY IdcA KapR OxyR BIlvY MetcA KapR OxysB IlvY MetcA KapR	EDHPWANI PDHPLAGI PALPCPU KSRTCTG EEHHLSSI NMVAAGS TYVRLGL SMVALGC QMVAARC NLVLNAD AMVSMGI .230 EAIRARMI QRFAPHL EAFWKIL	R EC K KA RNQVS K - TTT F - ST V882 USU USU USU USU USU USU USU US	VPMA ITIE VEKF VPLE 80 PALA ASMA PEVV PHWV PHWV ADSY 40 KVLK	AVP- AVP- AVP- AVP- AVP- AVP- AVP- AVP-	GEKF SET SET SET SET SET SET SET SET SET SET	LLI LLV FTI LLV FV 90 RKK PV FV FV SC FG MN 2 	MLET TYT IIYPQ TMP CTMP CDPDR QGD SNPG SNPG SNPG S0 	Second Se	· A66L9-CLGGVCRY HTTVRRYCACTON LVDEKPP IK - CO	RD(RRSI RRSI WDD 00 CII RRSI VEI - LI .2 - LI .2	AMDDEELR	GF TA WF HF TT RV SHPV IP	CFI FNI RRI LQI CQC RT ST SFEI SRI AQD	EAG RAG RAG RNG 2210 IGI IGI LGY LYY LYY	LUTION CONTRACTOR	CIPHASIC HELLS	ATT ATT ATT ATT ATT ATT ATT ATT ATT ATT	I S6124-FFASUR · PFF-QR- ·	C RAAVDTVCE22 LRRKQK	TTDGNSE SSYLVA S	LADELVQ. YMHESP.	TL VI AM LL TI VI AM UDF PLF AM	RKVIYL · AIIIIVV ·
DXYR CYSB IlvY IdvR IdcA XapR OCYSB IlvY MedcA XapR CYSB IlvY KapR OCYSB IlvY MedcA	EDHPWANI PDHPLAGI KSRTCTG EEHHLSSI NMVAAGS TYVRLGL SMVALGC QMVAARA NLVLNAD AMVSMGI .230 EAIRARMI QRFAPHL EAFWKIL RSARNHA	R EC K KA RNQVS K - TTT F - ST F - ST GITLLI GVGVI GVALL GIAAL GIAAL GIAAL GIAAL GIAAL GIAAL GIAAL GIAAL GIAL GI	VPMA ITIE VEKF VPLE 80 PALA ASMA PEVV PHWV ADSY 40 KVLK DAAV FVKS	ADDA ADDA ADDA ADDA ADDA ADDA ADDA ADD	GEKFURE QQYPTVFSET SEQUENT 	LLI LLV FII LLV FV 90 RK VA FV FV SVA FV FV SVA EI 	MLET TYADYQQ TMADYQQ TMA DDNRQQQQ 5 	Second Se	AG61-CLGSAGTD2 AG77VRSYD2 AG77VRS	RD(RRSI RRSI WDD) 00 CII RRSI VEI - LI .2 LI .2 	AMDLRLQ · PIERGETQ · .	GF TA WFF TT RV	CFI FRI LQI CQC RT: ST: SRI AQD	EAC RAC PAC 210 IGI ICI LGY LY LY LY		CIPHON HELLAND	CTI CTI CTI CTI CTI CTI CTI CTI CTI CTI		C RAAVDTVKE22 LRRKQKQ	TTGNDNO SSRPKQ28	LAHTVP · YMHTATO	TLI VIAM LLI TV ODFL AF	RKVIYL · AIIIVV ·
OXYR CYSB IlvYR MdcA XapR OCYSB IlvY MedcA XapR OCYSB IlvY MedcA XapR	EDHPWANI PDHPLAGI KSRTCTG EEHHLSSI NMVAAGS TYVRLGL SMVALGC QMVAARM NLVLNAD AMVSMGI .230 EAIRARMI QRFAPHL EAFWKIL RSARNHA ELAKYS KLLAALT	R EC K KA RNQVS K - TTT F - ST F - ST GITLLI GVGVI GVALL GIAAL GIAAL GIAAL GIAAL GIAAL GIALL GIAAL GIALL G	VPMA ITIE VEKF VPLES- VPLE 80 PALA ASMA PEVV PHWV ADSY 40 KVLK DAAV FVKS R.RRC	ADEA COMA COMA COMA COMA COMA COMA COMA COM	GEKFURES	LLU LV FII FV 90 RK VA FV FV S0 FC FG MN .2 EI 	MLET TYT MAD IYPQ IYPQ IYPQ IYPQ SO COLORN SNPQ SO SO SO SO SO SO SO SO SO SO SO SO SO	14 59 10 10 10 10 10 10 10 10 10 10	AG61-CLGSAGTC2 AG72VRRVLTXPPDCAGTC2 AG72VRRVLTXPPDCAGTC2 AG72VRRVLTXPPC AG72VRRVLTXPPC AG72VRRVLTXPPC AG72VRRVLTXPPC AG72VRRVLTXPPC AG72VRRVLT2 AG72VRT2	RD(RSI RRSI LD) WD) 000 CAHI RSI TL(VE: - L1 .2 L1 .2 	ANDLELC · PIEETQG · KDDGETQG · K	GFTA WFF RV PR SHF LW PV	CFI FRI LQI CQQ RT: SFEI SRI AQI	EAG RAG PAG RNG 2210 IGI IK: LGY LYY LYY LYY	LUI LUI LUI LUI LUI LUI LUI LUI LUI LUI	CIPHASIS - HELLAN SISTEMAN SIS	PG: SII SII SII SII SII SII SII SII SII SI		C RAAVDTVKE22 LLRKQKU	TTDG-SSYLVAV28	LEDELLVQ. YMHEESP.	TLL VI AM LLL TTV QLF PLF VLA 	RKVIYL · AIIIVV ·

FIG. 1. Alignment of *E. coli* LysR family members. The amino acid sequences of the *E. coli* OxyR, CysB, IlvY, MetR, TdcA, and XapA LysR-type transcriptional regulators were aligned by using CLUSTAL W software. Numbering is based on the OxyR sequence. Amino acids identical in five of the six proteins are highlighted in black, and conserved residues are highlighted with gray. Brackets denote regions mutated in the alanine-scan mutagenesis. Positions of activation-defective mutations isolated in the random mutagenesis screen are indicated above the sequence.

bond formation. To examine this possibility, we carried out DNase I footprinting experiments to assay T238A protein binding to the site between the divergent *oxyR* and *oxyS* promoters. Wild-type OxyR binds to this site in both its reduced and oxidized confirmations but makes different DNA contacts in the two conformations (25). The assays with purified T238A showed that, under oxidizing conditions, this mutant has a reduced footprint highly similar to the C199S mutant, which is locked in the reduced conformation (Fig. 3). This result suggests that T238 influences C199-C208 disulfide bond formation and that the T238A mutant is less readily oxidized. In contrast,

the purified D142A protein behaves more like the wild-type protein and shows a predominantly oxidized footprint under these conditions.

A negative charge of D142 is essential for gene activation by OxyR. D142 is a negatively charged residue. To test the importance of the charge of this residue, we examined the properties of mutants in which D142 was substituted by another negatively charged residue (E), neutral amino acids (N and Q), and a positively charged residue (K) (Table 3 and Fig. 4). The D142E mutant showed wild-type resistance to oxidants and wild-type induction of *oxyS* upon treatment with hydrogen per-

TABLE 2. Peroxide sensitivity of alanine-scan mutants

Plasmid	Codon exchange	Zone of inhibition $(mm)^a$ with:					
		10% H ₂ O ₂	4% CHP				
pACYC184		39	29				
pAQ5		22	17				
Q135A	<u>CA</u> A- <u>GC</u> A	21	17				
L136A	CTG-GCG	26	20				
D137A	GAC-GCC	22	18				
S138A	<u>AGC-GC</u> C	22	17				
G139A	GGC-GCC	25	18				
K140A	<u>AA</u> A- <u>GC</u> A	23	18				
L141A	<u>CT</u> C- <u>GC</u> C	22	17				
D142A	G <u>A</u> T-G <u>C</u> T	34	25				
V144A	G <u>T</u> G-G <u>C</u> G	23	18				
I145A	<u>ATC-GC</u> C	23	17				
pACYC184		38	29				
pAQ5		22	19				
V231A	G <u>T</u> G-G <u>C</u> G	22	19				
G234A	G <u>G</u> T-G <u>C</u> T	23	18				
S235A	<u>AG</u> C- <u>GC</u> C	23	17				
G236A	G <u>G</u> G-G <u>C</u> G	25	20				
I237A	<u>ATC-GC</u> C	23	16				
T238A	<u>ACT-G</u> CT	31	26				
L239A	<u>TT</u> A- <u>GC</u> A	22	15				
L240A	<u>CT</u> G- <u>GC</u> G	23	16				
P241A	<u>C</u> CA- <u>G</u> CA	24	20				

^{*a*} That is, the total diameter of the growth inhibition zone caused by the addition of hydrogen peroxide (H_2O_2) or cumene hydroperoxide (CHP). The values correspond to the average of three independent assays.

oxide. In contrast, the D142Q and D142N mutants had increased sensitivity and showed reduced ability to activate transcription, and the D142K mutant behaved like the $\Delta oxyR$ mutant strain, indicating a negatively charged residue is important in this position. The levels of the D142K mutant protein were elevated compared to the wild-type protein, although not to the extent observed for mutants carrying substitutions in



FIG. 2. Primer extension analysis of *oxyS* induction in alanine-scan mutants. Total RNA was isolated from the corresponding *E. coli* strains grown to mid-exponential phase and either left untreated (-) or exposed to 200 μ M hydrogen peroxide (+) for 5 min. A labeled oligonucleotide capable of hybridizing to the OxyS RNA was incubated with 5 μ g of each RNA sample and extended with reverse transcriptase.



FIG. 3. DNase I footprint analysis of purified D142A and T238A OxyR mutant proteins binding to an *axyS-axyR* promoter fragment. The 100-bp EcoRI-HindIII fragment of pGSO40 (25) labeled at the HindIII site (top strand relative to the *axyS* promoter) or EcoRI site (bottom strand relative to the *axyS* promoter) or EcoRI site (bottom strand relative to the *axyS* promoter) was incubated with 50, 160, 170, and 150 ng of purified wild-type, C199S, T238A, and D142A OxyR, respectively. Footprinting assays were carried out in the absence of dithiothreitol; a short oxidized footprint is observed for wild-type OxyR under these conditions.

the DNA-binding domain (see below). Thus, the D142K mutant may be somewhat defective in autorepression and DNA binding, and some of the lack of activation may be attributable to decreased promoter binding. However, the levels of the

TABLE 3. Peroxide sensitivity of D142 mutants

Diamaid	Cadan ambana	Zone of inhibition $(mm)^a$ with:						
Plasmid	Codon exchange	10% H ₂ O ₂	4% CHP					
pACYC184		38	27					
pAQ5		23	18					
D142A	GAT-GCT	32	24					
D142E	GAT-GAA	22	19					
D142N	<u>GAT-AAT</u>	33	26					
D142Q	GAT-CAG	31	24					
D142K	$\overline{\mathbf{G}}\mathbf{A}\overline{\mathbf{T}}$ - $\overline{\mathbf{A}}\overline{\mathbf{A}}\overline{\mathbf{A}}$	37	28					

 a That is, the total diameter of the growth inhibition zone caused by the addition of hydrogen peroxide (H₂O₂) or cumene hydroperoxide (CHP). The values correspond to the average of three independent assays.



FIG. 4. (A) Immunoblot of D142 mutant protein levels. The total protein corresponding to equal numbers of cells was separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed with α -OxyR antiserum. (B) Primer extension analysis of *oxyS* induction in D142 mutants. The total RNA was isolated from the corresponding *E. coli* strains grown to mid-exponential phase and either left untreated (–) or exposed to 200 μ M hydrogen peroxide (+) for 5 min. A labeled oligonucleotide capable of hybridizing to the OxyS RNA was incubated with 5 μ g of each RNA sample and extended with reverse transcriptase.

D142A, D142N, and D142Q mutants were similar to the wildtype levels, indicating that these activation-defective mutants are fully able to bind DNA.

Random mutagenesis screen for activation-defective mutants. In previous studies, we designed genetic screens for constitutive and for nonbinding OxyR mutants (7, 8). To screen for an activation-defective OxyR mutant, we used an *ahpCF* deletion strain, which lacks the alkylhydroperoxide reductase and thus has elevated endogenous levels of hydrogen peroxide (17). In this background the wild-type OxyR protein is constitutively in its oxidized (activated) conformation (15). The strain also was engineered to carry a prophage with a lacZfusion to the OxyR target oxyS, as well as a deletion of the chromosomal copy of *oxyR*. On MacConkey plates, the $\Phi(oxyS)$ *lacZ*) $\Delta oxyR$::Sp $\Delta ahpCF$::Kan strain (GSO132) carrying the pACYC184 control vector is white, whereas this strain carrying the pAQ5 plasmid encoding wild-type oxyR is red. On tetrazolium indicator plates, the GSO132/pACYC184 strain is red, and the GSO132/pAQ5 strain is white. pAQ5 was mutagenized by passage through a mutator strain or by treatment with hydroxylamine and transformed into GSO132. Plasmids were extracted from all white colonies identified on MacConkey plates and all red colonies identified on tetrazolium plates. The phenotypes were confirmed by reintroducing the plasmids into the same background. All of the mutants showing decreased oxyS-lacZ expression were tested for hydrogen peroxide sensitivity (Table 4). Immunoblots were also carried out to eliminate all mutants expressing truncated versions of OxyR (Fig. 5A and data not shown). The plasmids associated with hypersensitivity to hydrogen peroxide and expressing full-length OxyR were then sequenced.

Mutants are impaired in DNA binding, oligomerization, disulfide bond formation, and transcriptional activation. At least four categories of activation-defective mutants were expected: (i) mutants defective in DNA binding and therefore also defective in *oxyR* autoregulation, (ii) mutants altered in oligomerization, (iii) mutants in which oxidation and disulfide bond formation are affected, and (iv) mutants defective in the interaction with RNA polymerase. To distinguish between these possibilities, we carried out a number of assays (Table 4 and Fig. 5) and mapped the positions of the mutations on the crystal structures of the OxyR regulatory domain (Fig. 6).

Six mutations-R4C, Y8C, Y8H, A22V, P30L, and S33N-

Plasmid	Codon exchange	Zone of i (mm) ^a	nhibition with:	OxyR protein ^b	$oxyR-lacZ^c$	Interpretation		
		10% H ₂ O ₂	4% CHP					
pACYC184		38	28	No expression	380			
pAQ5		22	19	Wild type	91			
R4C	CGT-TGT	39	29	Overexpressed $(+++)$	350	DNA binding		
Y8C	TAC-TGC	36	28	Overexpressed $(+++)$	340	DNA binding		
Y8H	TAC-CAC	29	23	Overexpressed $(+++)$	350	DNA binding		
A22V	GCA-GTA	39	28	Overexpressed $(+++)$	360	DNA binding		
P30L	<u>C</u> GA- <u>T</u> GA	37	27	Overexpressed $(+++)$	350	DNA binding		
S33N	AGC-AAC	38	29	Overexpressed $(+++)$	350	DNA binding		
G96D	$G\overline{G}T-G\overline{A}T$	38	28	Overexpressed $(++)$	220	Oligomerization		
G102R	GGA-AGA	37	28	Overexpressed $(++)$	240	Oligomerization		
P103L	CCG-CTG	31	27	Wild type	33	Oligomerization		
H125P	CAT-CCT	32	27	Wild type	29	Oligomerization		
E126K	GAA-AAA	36	26	Overexpressed $(++)$	220	Oligomerization		
T129A	ACC-GCC	36	27	Wild type	22	Oxidation		
D196G	GAT-GGT	28	24	Wild type	24	Oxidation		
C199Y	TGT-TAT	30	18	Wild type	65	Oxidation		
F219S	TTC-TCC	37	27	Overexpressed (+)	47	Oligomerization		
T238A	ACT-GCT	30	25	Wild type	24	Oxidation		
R273H	$\overline{C}\underline{G}T$ - $\overline{C}\underline{A}T$	45	34	Overexpressed (++)	80	Positive control		

TABLE 4. Phenotype of uninducible mutants

 a That is, the total diameter of the growth inhibition zone caused by the addition of hydrogen peroxide (H₂O₂) or cumene hydroperoxide (CHP). The values correspond to the average of three independent assays.

^b Approximate amounts of OxyR proteins in Fig. 5.

^c Miller units of β-galactosidase activity. The values correspond to the average of two experiments.



FIG. 5. (A) Immunoblot of OxyR protein levels in activation-defective mutants. Total protein corresponding to equal numbers of cells was separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed with α -OxyR antiserum. (B) Primer extension analysis of α_{VS} induction in activation-defective mutants. Total RNA was isolated from the corresponding *E. coli* strains grown to mid-exponential phase and either left untreated (–) or exposed to 200 μ M hydrogen peroxide (+) for 5 min. A labeled oligonucleotide capable of hybridizing to the OxyS RNA was incubated with 5 μ g of each RNA sample and extended with reverse transcriptase.

mapped to the helix-turn-helix DNA-binding domain not present in the crystal structure. Immunoblots of all of the corresponding mutants showed highly elevated OxyR expression (Fig. 5A). These mutants also were unable to repress the *oxyR-lacZ* fusion (Table 4). Thus, we suggest that the R4C, Y8C, Y8H, A22V, P30L, and S33N mutants are defective in DNA binding. Two of these mutants, R4C and S33N, were previously isolated in the screen for nonbinding mutants (7).

An additional six mutations—G96D, G102R, P103L, H125P, E126K, and F219S—mapped to the interface between the two monomer subunits. Two of the corresponding mutants, P103L and G96D, had only minor defects in OxyS induction, and a third, H125P, was only partially defective (Fig. 5B). In contrast, no induction was detected for the G102R, E126K, and F219S mutants. Two of these stronger mutants, G102R and E126K, also had elevated OxyR levels (Fig. 5A) and increased *oxyR-lacZ* expression (Table 4), indicating that possible defects in oligomerization might be associated with defects in DNA binding. Interestingly, the G96D mutant had similar elevated levels of the OxyR protein and *oxyR-lacZ* expression but was far less defective in OxyS induction, raising the possibility that the



FIG. 6. Positions of constitutive and activation-defective mutations on the OxyR structure. Space-filling model of the OxyR regulatory domain in the reduced (A) and oxidized (B) conformations was generated by using InsightII software (Accelrys Software, Inc.). The front view is given on the left, and the back view is on the right. One monomer is shown in aqua, and the second monomer is shown in green. The positions of the C199 and C208 residues are indicated in yellow. The residues affected by the constitutive mutations (T100, H114, H198, R201, and A233) isolated in a previous screen for elevated expression of an *oxyS-galK* fusion (8) are indicated in red. The residues found to be mutated in the activation-defective mutants (G96, G102, P103, H125, E126, T129, D142, F219, T238, and R273) described here are indicated in orange. The black box surrounds residues D142 and R273.

G96D substitution actually enhances activation. Given the locations of the mutations, we suggest all six affect activation by altering the interaction between the monomers.

Four substitutions-T129A, D196G, C199Y, and T238Amapped at or near C199 and C208, the two redox-active cysteine residues. The C199Y mutation affects one of the two redox-active cysteines. The T129 residue touches C199, and the D196 residue is on the same loop as C199; it is likely that both of the corresponding mutations affect the reactivity of C199. The T238A mutation is equivalent to the mutation generated by the alanine-scan mutagenesis and is also thought to affect the reactivity of the redox-active cysteines. The T129, C199Y, and T238A mutant proteins had reduced footprints under oxidizing conditions (Fig. 2 and data not shown), a finding consistent with the suggestion that these mutants are impaired in disulfide bond formation. The T129A and T238A mutants are both hypersensitive to oxidants but still show some OxyS RNA induction. In contrast, the C199Y mutant is partially constitutively active, similar to what has been observed for a C208S mutant (9). The footprint of the D196G mutant protein was similar to the wild-type protein under oxidizing conditions. However, the mutation had minor effects; the D196G mutant was only partially sensitive to oxidants and only showed slightly reduced OxyS induction.

The remaining mutation, R273H, mapped to a surface-exposed residue and had a dramatic effect. Absolutely no OxyS induction was observed in this strain, indicating that the mutant is completely impaired in transcriptional activation. The R273H protein levels were somewhat elevated, though the expression of the *oxyR-lacZ* fusion was similar to the wild-type OxyR strain. We do not know the reason for the discrepancy between these two assays of autorepression, but the increased R273H levels may reflect a partial defect in DNA binding or altered sensitivity to degradation. Intriguingly, the R273H mutant was even more sensitive to both hydrogen peroxide and cumene hydroperoxide than the pACYC184 vector control strain, suggesting that expression of the cell.

D142 and R273 define a possible activating region. One mutant, D142A, isolated in our alanine-scan approach and another isolated in our random screen, R273H, show strong defects in OxyR activation of the oxyS target gene but only partial defects in DNA binding. Intriguingly, the two residues affected by the mutations are adjacent in the OxyR regulatory domain (Fig. 6). There are several possible explanations for the observed defect in transcription activation. D142A and R273H map to an interaction surface between the regulatory and DNA-binding domains of the structure of the full-length Ralstonia eutropha CbnR protein solved in the absence of inducer (14). Thus, the substitutions may be affecting transcription activation through some effects on DNA binding. The structure of a full-length LysR-type regulator in an inducerbound, activated conformation is not yet available. However, given that R273 maps onto the surface of the CbnR structure and both D142 and R273 have the potential to be exposed in the activated conformation, the two residues may also correspond to a point of contact between OxyR and RNA polymerase and thus define an activating region on the OxyR protein. Alternatively, the D142A and R273H may be altering the OxyR conformation such that the actual contact residues can no longer interact with RNA polymerase.

Activating regions are well defined for a number of transcriptional regulators that are not members of the LysR family (reviewed in references 2 and 5). For example, the E34 and D38 residues of the λcI activator have been shown to contact the R588 and R596 residues of the σ^{70} subunit of RNA polymerase. Similarly, the D241 residue of the *E. coli* RhaS protein, a member of the AraC/XylS family of transcriptional regulators, interacts with R599 of σ^{70} . Multiple activating regions that interact with both the α and σ^{70} subunits of RNA polymerase also have been defined for the *E. coli* CRP activator; ARI (T158), ARII (H19, H21, D21, and K101) and ARIII (D53, E54, E55, and E58). It is notable that many of the residues shown to be involved in contacting RNA polymerase are charged, as is the case for D142 and R273.

Less is known about possible activating regions for LysRtype transcriptional regulators. Two studies propose that residues in the DNA-binding domain of these transcription factors contact the α subunit of RNA polymerase. L30A, F31A, and F32L substitutions in the *E. coli* GcvA regulator result in reduced activation of the *gcvT* promoter but do not reduce DNA binding or autorepression (6). Similarly, derivatives of the *E. coli* CysB protein with substitutions of the Y27, T28, and S29 residues are defective for activation of the *cysP* promoter but not for inducer or DNA binding (10). It is possible that LysR family members have multiple activating regions, as is the case for CRP. The GcvA protein has in fact been shown to interact with both α and σ^{70} subunits of RNA polymerase (19).

The strongest support for the model that the D142 and R273 residues act as a contact point for RNA polymerase would come from the isolation of suppressor mutations in subunits of RNA polymerase, as has been the case for some of the non-LysR regulators described above. All genetic screens to isolate such suppressors for the putative OxyR positive control mutants thus far have been unsuccessful (data not shown), and no such suppressors have been reported for other LysR family members. Thus, additional biochemical and structural studies of LysR-type transcriptional regulators will need to be carried out before the precise contacts between this family of transcription factors and RNA polymerase can be defined.

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