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OxyR: A Molecular Code for Redox-Related Signaling

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Summary

Redox regulation has been perceived as a simple on-off switch in proteins (corresponding to reduced and oxidized states). Using the transcription factor OxyR as a model, we have generated, in vitro, several stable, posttranslational modifications of the single regulatory thiol (SH), including S-NO, S-OH, and S-SG, and shown that each occurs in vivo. These modified forms of OxyR are transcriptionally active but differ in structure, cooperative properties, DNA binding affinity, and promoter activities. OxyR can thus process different redox-related signals into distinct transcriptional responses. More generally, our data suggest a code for redox control through which allosteric proteins can subserve either graded (cooperative) or maximal (noncooperative) responses, and through which differential responsivity to redox-related signals can be achieved.

Introduction

Reactive oxygen and nitrogen species are at the crux of the pathogenesis of disparate disorders, including tissue inflammation, chronic infection, malignant transformation, and degenerative diseases (Nakamura et al., 1997). Cells combat this common form of insult by activation of redox-response genes (Sen, 1998; Stamler et al., 2001). These genes encode enzymes involved in detoxification of the reactive molecules, export, repair, and other homeostatic functions. A number of different classes of transcription factors are implicated in such control of gene expression (Marshall et al., 2000). Although our understanding of redox regulation of these proteins is still rudimentary, well-studied examples demonstrate that cysteine residues seem to serve sensory and regulatory roles in the activation mechanism (Abate et al., 1990; Demple, 1998; Sen, 1998; Marshall et al., 2000). That is, cysteine redox centers in transcriptional activators typically confer responsiveness to nitric oxide (NO) and oxygen (O_2)-related molecules (Marshall et al., 2000; Stamler et al., 2001). However, the operation of adaptive mechanisms that compensate for oxidative or nitrosative stress has not been clearly differentiated from physiological, signal- and target-specific redox regulation in these systems, and both the identity and mechanism of the function-regulating protein modifications in situ remain elusive (Sen, 1998; Marshall et al., 2000; Irani, 2000).

The prototypic cysteine redox center involves thiol residues that can be oxidized to intramolecular or mixed disulfides (Gilbert, 1990; Hutchison et al., 1991; Ruppersberg et al., 1991; Lipton et al., 1993; Pineda-Molina et al., 2001; Choi et al., 2001). Reduced thiols constitute the resting (e.g., transcriptionally inactive) condition, whereas disulfide formation provides the activation mechanism or "switch." This model, which has emerged from studies of paradigmatic redox systems such as OxyR (the bacterial transcriptional activator of *E. coli*), is well adapted to serve in sensing global redox status of the cell, as in oxidative stress (Demple, 1998; Zheng et al., 1998), but does not supply an obvious mechanism for the differential and graded responsivity that is characteristic of regulated signal transduction. In particular, current models do not provide for the possibility of differential recognition of redox-related signaling molecules that would subserve distinct effects, and thus do not provide for the specificity or sensitivity that characterize physiological signal transduction networks.

OxyR belongs to a family known as the LysR type (Schell, 1993) of DNA binding proteins, characterized by the positive regulation of target genes and the negative regulation of their own expression. These transcription factors, including OxyR, bind DNA either as dimers or tetramers using a helix-turn-helix motif of binding, with each monomer binding on an adjacent part of the DNA duplex (Tartaglia et al., 1992; Choi et al., 2001). Five residues are conserved in the DNaseI protected regions of OxyR binding sites which otherwise appear to lack a consensus or recognition motif (Tartaglia et al., 1992). The reduced form of OxyR has DNA binding activity but does not generally activate transcription. In response to hydrogen peroxide or S-nitrosothiols, OxyR induces the expression of multiple genes that protect from both oxidative and nitrosative stress, as well as *oxyS* (a gene encoding a nontranslated RNA involved in DNA repair; Hausladen et al., 1996; Altuvia et al., 1997). Genetic, biochemical, and structural studies suggest that an intramolecular disulfide subserves the role of the redox switch (Zheng et al., 1998; Choi et al., 2001). The thiol-disulfide redox status of the cytosol is thus proposed to regulate the activation of OxyR (the redox potential of OxyR is more positive than the resting cytosol by 95 mV). A number of features of the proposed mechanism are, however, difficult to reconcile. First, only one thiol in OxyR is absolutely critical for activity, as well as necessary and sufficient for wild-type phenotype (Storz et al., 1990; Kullik et al., 1995b). Second, a single, stable S-nitrosothiol (SNO) has been identified in OxyR (Hausladen et al., 1996), whereas it should rapidly convert to intramolecular disulfide if vicinal thiols were present (Gravina and Mieyal, 1993; Arnelle and Stamler, 1995;

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Becker et al., 1998). Third, activation of OxyR by hydrogen peroxide and S-nitrosothiol is not equally reversible (Hausladen et al., 1996). Finally, redox activators of SoxR do not activate OxyR, even though these effector molecules can alter cellular redox state.

The common perception of redox regulation as a simple "on-off switch" (exemplified in thiol/disulfide interconversion) is also inconsistent with accumulating data. DelaTorre et al. (1997) noted, for example, that the DNA binding affinity of an NO-treated form of NFκB was modestly decreased, whereas the binding of an alternatively oxidized form was virtually eliminated (Brennan and O'Neill, 1995). In addition, we have demonstrated that cysteine redox centers can in fact differentiate nitrosative from oxidative events (Xu et al., 1998; Eu et al., 2000). Furthermore, it is known that cysteine thiols are subject to multiple redox-based modifications that may alter protein function (Allison, 1976; Gilbert, 1990; Stamler and Hausladen, 1998). Collectively, the data suggest an alternative model in which diverse cysteine modifications are generated by alternative physiological signals, and elicit different functional responses (Stamler and Hausladen, 1998). This alternate theory holds not only that proteins can discriminate among redox-related signaling molecules, but also that different thiol modifications can have unique functional effects (Stamler and Hausladen, 1998). It predicts that cysteine modification by *S*-nitrosylation (S-NO), or by oxidation to form either a mixed disulfide with glutathione (S-SG, *S*-glutathionylation), an intramolecular disulfide (S-S), or a sulfenic acid (S-OH, *S*-hydroxylation), may be alternatively exploited to control gene expression.

The main reason that the mechanisms governing redox regulation of cell signaling remain largely speculative is that the candidate redox-related target modifications have not been produced in pure form for any protein and thus, structure-function analyses have not been possible. Inherent instability and mutability of the redox modifications over the time course of analysis are additional obstacles that have not been addressed. We have now produced four stable, redox-related modifications in the transcriptional activator OxyR, identified them in vivo, and characterized their differential effects on structure, DNA binding, and transcription. These observations provide us with the basis of a code that relates structure to function and thus with the opportunity to unravel the diverse mechanisms of redox regulation. Our results show that cooperative effects in proteins can be redox based, and suggest not only the possibility of graded responses to alternative redox-related signals, but also that switching mechanisms can exhibit a high degree of selectivity and specificity, enabling different redox-related signals to have qualitatively distinct effects; in this case, the differential transcriptional control of multiple genes.

Results

Background

Out of six cysteine residues per OxyR monomer, only C199 is essential for activity (Kullik et al., 1995b). Specifically, *E. coli* harboring the C199 to Ser (C199S) mutation is hypersensitive to hydrogen peroxide and the protein is transcriptionally inactive in vitro, even under oxidizing conditions. Optimal transcriptional activity, however, also requires C208. In particular, the phenotype of the C208S mutant is similar to the wild-type strain, but there is lower induction of transcription. Our studies with mutant OxyR proteins (C199S or C208S substitutions) generally confirmed these results (see below).

Characterization of OxyR Cysteines

C199 and C208 are involved in transcriptional activation, but the role and reactivity of the four remaining cysteines are unknown. We therefore characterized OxyR cysteines by quantitative amino acid analysis and mass spectrometry of protein labeled with iodoacetamide (IA) under various reducing and denaturing conditions. Specifically, carboxymethylcysteine (CM-Cys) was quantified by amino acid analysis, and the modified residues were identified by mass spectrometry following tryptic digests. When native, air-oxidized OxyR (as purified from *E. coli*) was treated with IA, 2 CM-Cys/mole OxyR were found in protein hydrolysates. Treatment of OxyR with IA in the presence of 6 M guanidine increased the yield to 4 CM-Cys per monomer of OxyR. The further addition of 10 mM DTT (i.e., 6 M guanidine + 10 mM DTT) yielded the expected 6 CM-Cys. Thus, we conclude that in OxyR: (1) two thiols are solvent-accessible (although one has low reactivity and can only be modified by IA; see below); (2) two free thiols are buried in the native protein and thus inaccessible to thiol reagents; and (3) two cysteines are involved in a disulfide bridge. This disulfide is accessible to reductants in the native protein, as 4 CM-Cys are recovered when 10 mM DTT is added without a denaturing agent.

To identify the various cysteine residues, tryptic digests of IA treated OxyR were analyzed by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF). All of the expected peptides (mass >500) were detected. The IA-modified cysteine-containing peptides are listed in Table 1A. In native "air oxidized" OxyR (as purified from *E. coli*), all IA-modified peptides mapped exclusively to either C199 or C25. In denatured OxyR, four IA-modified cysteine-containing peptides corresponding to C143, C199, C25, and C208 were detected. In addition, a peptide of m/z 2845.2 was found, in good agreement with the expected mass (2844.4) for two disulfide-linked peptides containing C180 and C259. All six expected cysteine-containing peptides were present in denatured and DTT treated OxyR. Thus, taken with the results obtained by amino acid analysis, we conclude that C25 and C199 are solvent-accessible thiols; that C143 and C208 are free thiols, albeit buried in the protein; and that C180 and C259 form a disulfide, which is accessible to reductants in the native (aerobically purified) state. These results are both supported by and add significantly to the recently published crystal structure of a 4Cys→Ala mutant form of OxyR (Choi et al., 2001). First, C199 is indeed solvent exposed, consistent with its high reactivity (Zheng et al., 1998; Choi et al., 2001), whereas C143 is buried within a β sheet that is enclosed between two α helices. No information on C25 can be gleaned, however, as the crystallographic data is derived from a truncated protein starting at residue 80. Second, the finding that residues C180 and C259

Table 1. Mass Spectrometry of OxyR

A

Residues	Sequence	Cys	m/z Expected	1	2	3	4
141–150	LDCVILALVK	C143	1143.7		1143.5	1143.4	
179–190	ECVPMADLAGEK	C180	1319.6			1319.4	1319.4
191–201	LLMLEDGHCLR	C199	1356.7	1356.8	1356.5	1356.5	1356.6
252–265	DGVVYLPCIKPEPR	C259	1642.9			1642.6	1642.7
21–37	AADSCHVSQPTLSGQIR	C25	1826.9	1827.2	1826.6	1826.6	1826.7
202–220	DQAMGFCFEAGADEDTHFR	C208	2203.9		2203.6	2203.5	
179–265		C180–C259	2845.4		2845.2		

B

Derivative	Expected M^{2+}	D^{3+}	$M^{+}D^{2+}$	D^{+}	Found (%) M^{2+}	D^{3+}	$M^{+}D^{2+}$	D^{+}
Native								
OxyR-SH	17318	22851	34276	68551	17136 (28)	22848 (8)	34272 (100)	68543 (45)
OxyR-SOH	17146	22861	34292	68583	17146 (7)	n.d.	34292 (20)	68580 (18)
NBD								
OxyR-SH	17138	22851	34276	68551	17132 (20)	n.d.	34264 (33)	68527 (23)
OxyR-SNBD	17220	22956	34440	68879	17217 (22)	22956 (18)	34433 (100)	68867 (55)
OxyR-S(O)NBD	17228	22971	34456	68911	17227 (10)	22956 (18)	34455 (33)	68912 (22)
SSG								
OxyR-SH			34276		n.d.	n.d.	34276 (28)	n.d.
OxyR-SSG			34581		n.d.	n.d.	34581 (40)	n.d.

(A) MALDI TOF mass spectrometry of tryptic digests of iodoacetamide (IA)-treated OxyR under (1) native (air oxidized OxyR + IA), (2) denaturing (air oxidized OxyR + 6M guanidine + IA), (3) denaturing and reducing (air oxidized OxyR + 6M guanidine + DTT + IA), and (4) native and reducing (reduced OxyR + IA) conditions. (B) Electrospray mass spectrometry of OxyR and its NBD and glutathione (SSG) derivatives. Expected m/z values for monomer (M) and dimer (D) are shown to the left and the measured values to the right. The number of charges is indicated by the superscript. Values in parentheses are the peak intensities relative to the highest peak for each spectrum. n.d.: not detected.

(alanines in the crystal) participate in a disulfide bond is supported by their very close proximity (5.9 Å). Third, the crystallographic data places distantly located C199 and C208 in a disulfide (despite being 18 Å apart in the truncated 4Cys→Ala mutant protein). We find no evidence for a disulfide bond between C199 and C208 in the full-length wild-type OxyR.

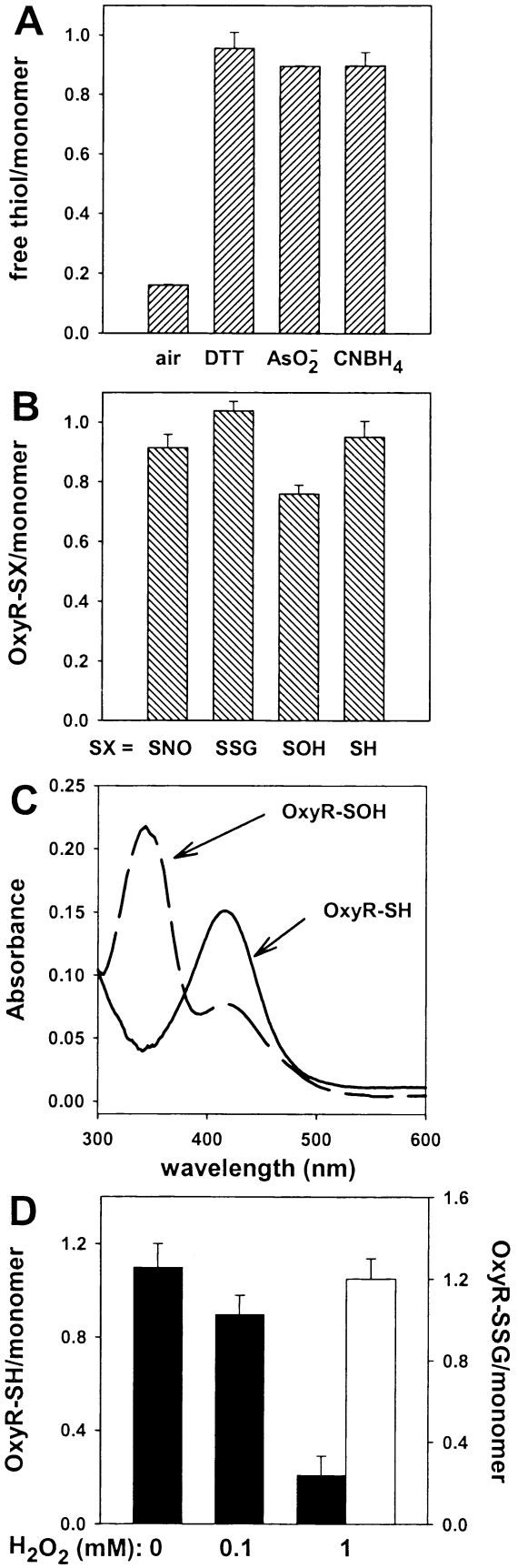
Thiol Modifications in OxyR

Synthesis and Characterization

We carried out thiol titrations in wild-type OxyR by several different methods. Only one SH was detected in the reduced protein as assessed by two standard assays (see below). This cysteine was identified as C199 by both the mass spectrometry studies and mutagenic analyses described below, confirming previous results (Storz et al., 1990; Kullik et al., 1995b; Zheng et al., 1998). We then undertook a characterization of the oxidation state of the air-activated form of native OxyR as well as the nature of the alternative forms produced from reactions with physiological effectors; i.e., S-nitrosothiols (SNOs), glutathione disulfide (GSSG), and hydrogen peroxide (H_2O_2).

OxyR-SOH

Air oxidized OxyR, the transcriptionally active form obtained by standard purification (Storz et al., 1990; Hausladen et al., 1996), contains 0.15–0.8 free thiol per monomer depending on the preparation (Figure 1A). Reduction with DTT or cyanoborohydride yielded 1 thiol per monomer of protein. A stoichiometry of one SH/OxyR monomer was also obtained by treatment with sodium arsenite (10 mM), a reducing agent specific for sulfenic acids (Torchinsky, 1981; Radi et al., 1991). The identification and stoichiometry of sulfenic acid was additionally verified using the reaction with dimedone (DeMaster et al., 1995; data not shown) and by reaction with the reagent 7-chloro-4-nitrobenzo-2-oxa-1, 3, diazole (NBD), as described by Ellis and Poole (1997; Figure 1C). NBD forms adducts with cysteine thiol (OxyR-SH) and cysteine sulfenic acid (OxyR-SOH) that absorb at 420 nm and 347 nm, respectively (Figure 1C). Thus, using three different chemical methods, we have established that the activated form of OxyR is sulfenic acid. To further verify these results, air oxidized native and NBD derivatized forms of OxyR were analyzed by electrospray mass spectroscopy (ESI-MS; Table 1B). For the native protein, peaks were found at 34272, corresponding to OxyR-SH (expected: 34276), and at 34292, corresponding to OxyR-SOH (expected: 34292). An additional peak was found at 34102, which likely results from fragmentation of two C-terminal residues (expected: 34106), as well as weaker peaks for OxyR-SH and OxyR-SOH dimers at 68543 (expected: 68552) and 68580 (expected: 68584), respectively. NBD derivatized OxyR produced peaks at 34264 (OxyR-SH, expected: 34276), 34433 (OxyR-SNBD, expected: 34440), and 34455 (OxyR-S(O)NBD, expected: 34457). Peaks for the dimer were at 68527, 68867, and 68912, corresponding to OxyR-SH (expected: 68551), OxyR-SNBD (expected: 68879), and OxyR-S(O)NBD (expected: 68911), respectively. Taken with the biochemical studies (above), these data firmly establish the presence of a sulfenic acid in native, air oxidized OxyR.



OxyR-SH

OxyR-SOH was reduced to OxyR-SH with DTT (200 mM) and desalted under strictly anaerobic conditions (see Experimental Procedures). In contrast to IA, which labeled two cysteines (Table 1), monobromobimane and Saville assays (Saville, 1958) detected only one free thiol per OxyR monomer (Figure 1B), consistent with the sulfenic acid quantification above. ESI-MS showed an m/z value of 34272 for the reduced protein monomer (OxyR-SH) (expected: 34276), and 17136 and 68543 for a double charged monomer and dimer, respectively (Table 1). The discrepancy between IA versus MBBr, dimedone, or Saville methods is probably due to low reactivity of one of the thiols. Indeed, IA labeled only one thiol under less stringent conditions (data not shown). MALDI-TOF of digests with chymotrypsin located an NBD thiol adduct on a peptide containing C199 (MLEDGHC(NBD)L, expected: 1081.5; found: 1082.2). Taken together with the mutagenic analyses detailed below, our data indicate that wild-type OxyR contains two free thiols, C199 and C25, but only C199 is reactive toward multiple modifying agents.

OxyR-SNO

Reduced OxyR (OxyR-SH) was treated with S-nitrosoglutathione (GSNO) or S-nitrosocysteine (CSNO), desalted anaerobically, and analyzed for SNO, as previously described (Hausladen et al., 1996). The OxyR derivative formed from GSNO contained one SNO per monomer (Figure 1B) as determined by two different assays (see Experimental Procedures). Treatment of OxyR-SNO with DTT regenerated OxyR-SH, whereas sodium arsenite had no effect. OxyR-SNO was stable in air for several days at 4°C. Definitive ESI-MS identification of SNO-OxyR could not be obtained.

OxyR-SSG

OxyR-SH exposed to a 10-fold excess of glutathione disulfide (GSSG) under strictly anaerobic conditions yielded the pure mixed disulfide of OxyR (OxyR-SSG); that is, OxyR containing one glutathione molecule per monomer (Figure 1B; see Experimental Procedures). OxyR-SSG could be reduced with DTT, but not with sodium arsenite. OxyR-SSG was stable for at least several days. ESI-MS identified both the OxyR-SSG adduct (34581; expected: 34581) and reduced OxyR-SH (34276; expected fragment: 34276; Table 1B).

Molecular modeling (Choi et al., 2001) suggests that OxyR has been adapted to serve in sensing GSSG (Figure 2). A SYBYL-generated (Tripos Inc., St. Louis, MO) Connolly surface (Connolly, 1983) in the locality of C199 in the crystal structure of the reduced form of OxyR (PDB

Figure 1. Characterization of Free and Modified Thiols of OxyR In Vitro and In Vivo

(A) Quantification of free thiol in air-purified OxyR and OxyR reduced anaerobically with either DTT (200 mM), sodium arsenite (10 mM), or cyanoborohydride (10 mM).

(B) Quantification of native and alternatively modified thiols in OxyR (see Experimental Procedures for details).

(C) Stable sulfenic acid in OxyR. Reaction of air-oxidized OxyR with NBD (dashed line): Cys-S(O)-NBD absorbtion at 347 nm; the shoulder at 420 nm is residual thiol (also seen in [A]). NBD reacted anaerobically with reduced OxyR (solid line): Cys-S-NBD absorbtion at 420 nm.

(D) In vivo modification of OxyR thiols. Cells overexpressing OxyR were treated with H_2O_2. In situ amounts of Oxy-SH (filled bars) and OxyR-SSG (open bar) are shown.

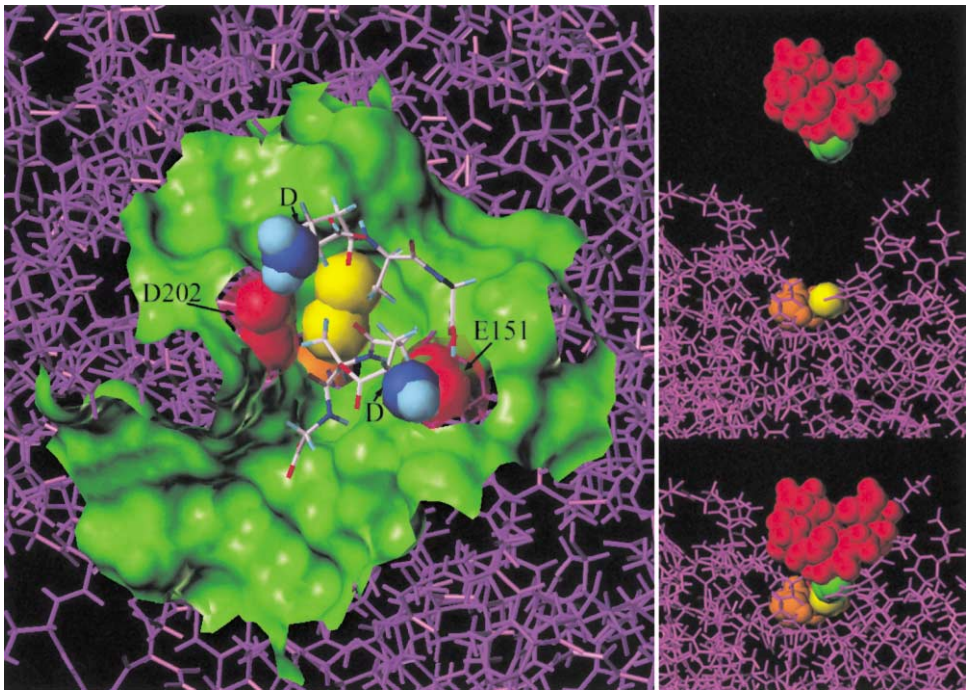


Figure 2. A Structural Model of GSSG Binding to Reduced OxyR

C199 resides in a pocket of relative hydrophobicity, surrounded by acids and bases; GSSG is docked with the disulfide bond 2 Å from the sulfur in C199. The interaction of GSSG is stabilized by two hydrogen bonds formed between amines of each glutathione and both E151 and D202. Atoms forming interactions are highlighted as space-filling models (sulfur: yellow/orange; nitrogen: blue; oxygen: red). A putative C199 S-nitrosylation motif is provided by the adjacent base (H198 or H130) and the candidate acids above (D and E); glutathione itself may also provide a proximal acid in quaternary structure. In the space-filling model of glutathione disulfide docking (right), GSSG is red and the sulfur is shown in green.

ID 1I69) shows a pocket (ca. 10 Å diameter) directly above C199. FlexX docking of GSSG (Rarey et al., 1996) produces a snug fit, with the disulfide bond in GSSG only 2 Å removed from the thiol in C199. In addition, the two amino groups of GSSG dock so that they form hydrogen bonds with two carboxylates of the protein (E151 and D202) located on opposite sides of the pocket rim.

H_2O_2-Oxidized OxyR

Reduced OxyR was exposed to a range of H_2O_2 concentrations (H_2O_2:OxyR, 1:10 to 100:1) for varying intervals (from minutes to 24 hours) under anaerobic conditions. Chemical analyses generally indicated the presence of a mixture of oxidized products, including sulfenic acid (arsenite/DTT reversible product) and higher oxides of sulfur (DTT/arsenite irreversible products). However, we found no evidence for intramolecular disulfide (i.e., a DTT or borohydride reversible/arsenite irreversible state), and were unable to fully oxidize the single free cysteine in buffers containing metal chelators.

In Situ Thiol Modification

We have thus far shown that native OxyR has a single accessible and reactive thiol (C199), and that this sensor cysteine subserves four redox-related states in vitro (SH, SOH, SNO, and SSG). In previous studies, we demonstrated that the SNO modification is produced in vivo upon exposure of *E. coli* to cytostatic concentrations of S-nitrosothiols (Hausladen et al., 1996), and we verified that we could purify OxyR containing up to 1 SNO/monomer from GSNO exposed cells (data not shown). We have also shown that air exposure can produce the sulfenic acid modification (Figure 1B). Next, we addressed whether the SH and SSG modifications of OxyR form in the native cytoplasmic milieu. To identify OxyR-SH in situ, we labeled cellular thiols in intact *E. coli* with the cell-permeant fluorescent tag, MBBr, purified the wild-type OxyR protein, and quantified OxyR labeling by fluorescence. This approach yielded a stoichiometry of 1.1 labeled thiol/OxyR monomer, indicating that OxyR is fully reduced in its native cellular environment (Figure 1D). When cells were treated with OxyR-activating amounts (0.1–1 mM) of H_2O_2, the reactive thiol was fully oxidized and varying amounts of glutathione mixed disulfide were formed (i.e., DTT liberated up to 1 GSH/OxyR monomer; Figure 1D). MBBr did not label OxyR derived from H_2O_2-treated cells, but did so efficiently when cells were either first exposed to DTT or exposed following aerobic purification ($\sim$1 MBBr/ OxyR). Thus, the H_2O_2-induced oxidation of OxyR in situ is reversible. Collectively, the data establish that the OxyR-SSG modification is produced in the activated form of OxyR in vivo (Figure 1D). Since we had noted (see above) that yields of sulfenic acid in aerobically purified OxyR varied among preparations, we assayed several such preparations for incorporation of glutathione (GS), finding as much as 0.5 GS incorporated, presumably due to oxidation during cell lysis (not shown). In conclusion, post-

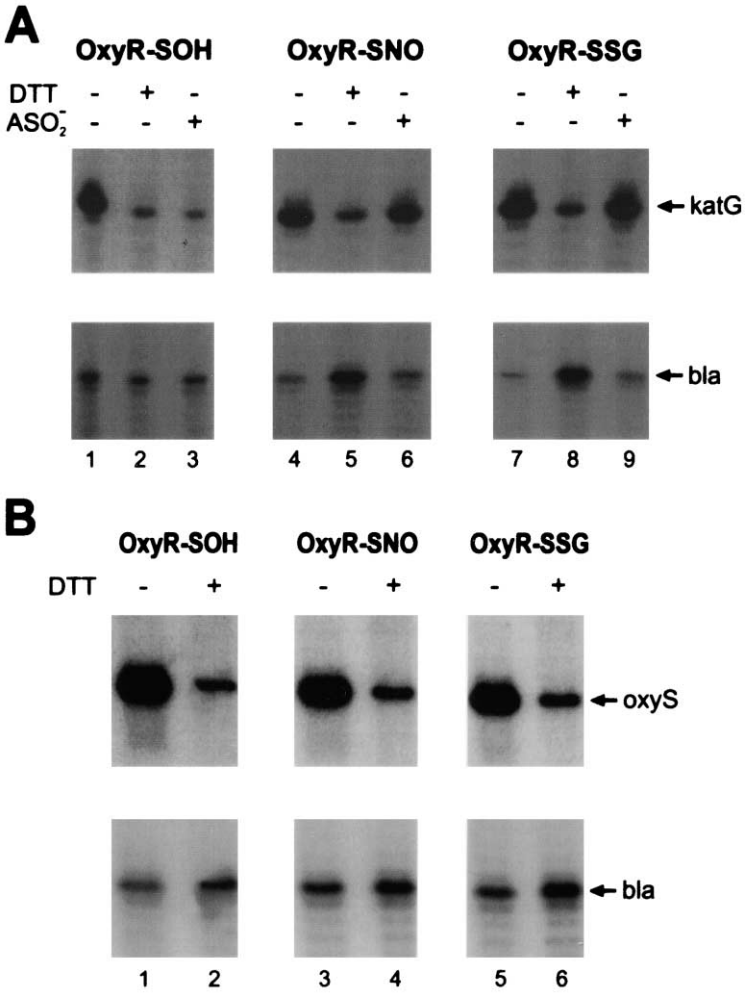


Figure 3. In Vitro Transcription by Redox-Related Forms of OxyR

(A) Primer extension products of in vitro transcription reactions performed with a plasmid carrying both the *katG* and β-lactamase (*bla*, control) genes. Lanes 1, 4, and 7 are without reducing agents. Lanes 2, 5, and 8 contain 200 mM DTT, and lanes 3, 6, and 9 contain 10 mM sodium arsenite (sulfenate-specific reductant). The activity of OxyR-SOH is decreased by both reducing agents, whereas the activity of OxyR-SNO and OxyR-SSG is only attenuated by DTT.

(B) Transcription of the *oxyS* gene. Generation of OxyR-SH with DTT reduces transcriptional activity uniformly.

translational modifications of OxyR-SH to SNO, SOH, or SSG occur in vivo in settings of nitrosative and oxidative stress.

Thiol Modifications in Mutant OxyR (C199S and C208S)

Reaction of reduced and air oxidized mutant proteins with NBD produced NBD adducts with cysteine thiol and cysteine sulfenic acid, respectively; i.e., both mutants showed characteristic absorbances at 420 nm and 347 nm (not shown). Surprisingly, biochemical analysis of mutant proteins (OxyR-SH) following exposure to GSSG revealed the formation of 1 glutathione mixed disulfide (OxyR-SSG) per monomer in both cases, whereas neither C199S nor C208S mutants could be S-nitrosylated by GSNO or CSNO ($\leq$ 0.2 SNO group per monomer). These results suggest that complex structural changes occur in the protein when either one of the cysteines is mutated. The mechanism for the unprecedented involvement of 2 thiols in producing a stable SNO is currently under investigation.

Transcriptional Activity

The activity of the native and modified forms of OxyR was assessed by in vitro transcription assays (Hausladen et al., 1996; Figure 3). A major problem in determining the functionality of a redox-related modification is that a sample may be contaminated by small amounts of other redox-related species. To overcome this pitfall, we conducted transcription assays in the presence and absence of reducing agents with differential effects on various redox-related species. Sodium arsenite is a reducing agent specific for sulfenic acid, whereas DTT reduces SNO, disulfides, and SOH, but not the higher oxides, SO_2^- and SO_3^-. Figure 3A shows that transcriptional activation of *katG* by OxyR-SOH (lanes 1 and 2), OxyR-SNO (lanes 4 and 5), and OxyR-SSG (lanes 7 and 8) was decreased by DTT; however, only OxyR-SOH activity was decreased by sodium arsenite (compare lanes 3, 6, and 9). Activation of the *oxyS* gene by modified forms of OxyR was also reversed by DTT (producing OxyR-SH; Figure 3B). Taken together these data: (1) verify that the air-oxidized form of OxyR (active prototype) is a sulfenic acid, and (2) establish that OxyR-SH, OxyR-SOH, OxyR-SNO, and OxyR-SSG are functionally distinct forms.

Molecular Basis of Differential Responsiveness

Secondary Structure Variations

Circular dichroism studies revealed unique features associated with each redox-related form of OxyR (Figure

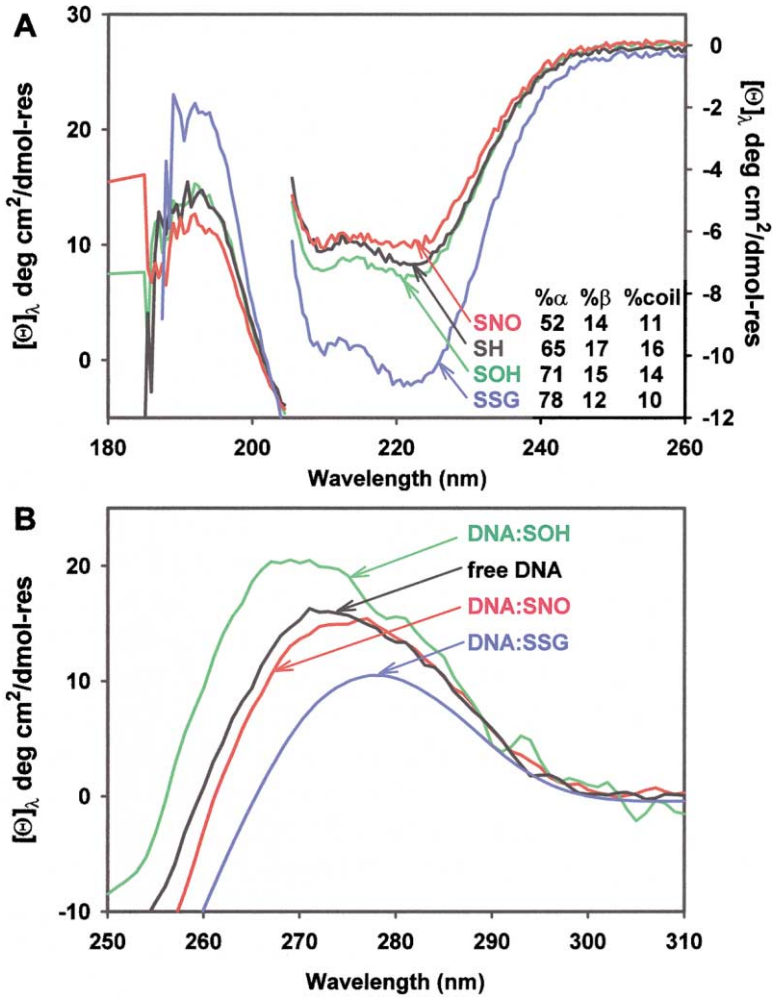


Figure 4. CD Spectra of OxyR and OxyR-Bound DNA

(A) OxyR and its redox-related forms. OxyR-SH (black), OxyR-SOH (green), OxyR-SNO (red), and Oxy-SSG (purple). Left Y axis is used for wavelengths 185-205 nm and right Y axis for wavelengths above 205 nm. Inset: % α helix, β sheet, and random coil (see Experimental Procedures).

(B) Bound and free DNA. The spectra of DNA (representing the katG binding site) bound to the different redox-related forms of OxyR are either blue-shifted (DNA:SOH), or red shifted (DNA:SNO and DNA:SSG) versus free DNA . OxyR protein does not show a CD signal at these wavelengths. The conformational changes in the DNA produced upon OxyR binding are thus different for each redox-related form.

4A). The greatest differences were seen at 222 nm, a wavelength at which the peak negative molar ellipticity is used as a measure of the α-helical content (Saxena and Wetlaufer, 1971). The CD spectrum of OxyR-SSG displays mostly α-helical characteristics. The other redox-related forms have reduced α-helical content (lower negative ellipticities at 222 nm and a red shift in the large positive peak below 200 nm). Each form, however, has distinct structural features (Figure 4A), which may impact the binding of the DNA substrate. Indeed, the active forms of OxyR also engender distinct variations in DNA structure (Figure 4B). More specifically, OxyR-SOH induced a distinct blue shift in the spectrum of a 48 base pair DNA oligonucleotide representing the *katG* binding site. On the other hand, the OxyR-SNO and OxyR-SSG-bound DNA spectra showed red shifts, to differing degrees. These variations were paralleled by differences in the binding properties of each redox-related form (see below), and may indicate protein interaction at distinct contact points along the DNA.

Functional Studies

Only oxidized OxyR binds *katG, ahpC, dps*, and *gorA*, whereas both the oxidized and reduced protein bind the *oxyRS* promoter (Toledano et al., 1994). We performed gel shift assays with the four redox-related forms of OxyR using promoters of both types: a 106 bp fragment containing the *katG* binding site and a 101 bp fragment containing the *oxyS* binding site. The fraction bound for a given OxyR modification was determined at concentrations ranging from 0.25 nM to saturation (approximately 10 nM). Our sedimentation equilibrium studies (not shown) indicated no significant change in dimer to tetramer ratio at these concentrations for any of the OxyR forms. (Dimer↔tetramer equilibrium constants for native and modified forms were found in the micromolar range, suggesting that dimeric species exist in solution, as previously reported by Tartaglia et al., 1992, and consistent with data which we interpret as showing a shift in equilibrium position toward dimer in the C199S mutant, which is locked in the reduced state; Kullik et al., 1995a.) The binding isotherms obtained with the different modified forms of OxyR and their Hill coefficients are shown in Figure 5A and 5B. The results reveal: (1) high affinity, noncooperative binding of OxyR-SH to the *oxyS* promoter and very weak, if any, binding to *katG*; (2) cooperative binding of OxyR-SNO to both promoters, albeit S-nitrosylation increases OxyR affinity for the *katG* promoter and decreases affinity toward *oxyS* (indicating relative selectivity for *katG*); (3) strong cooperative binding of OxyR-SOH to both promoters (*katG* in particular),

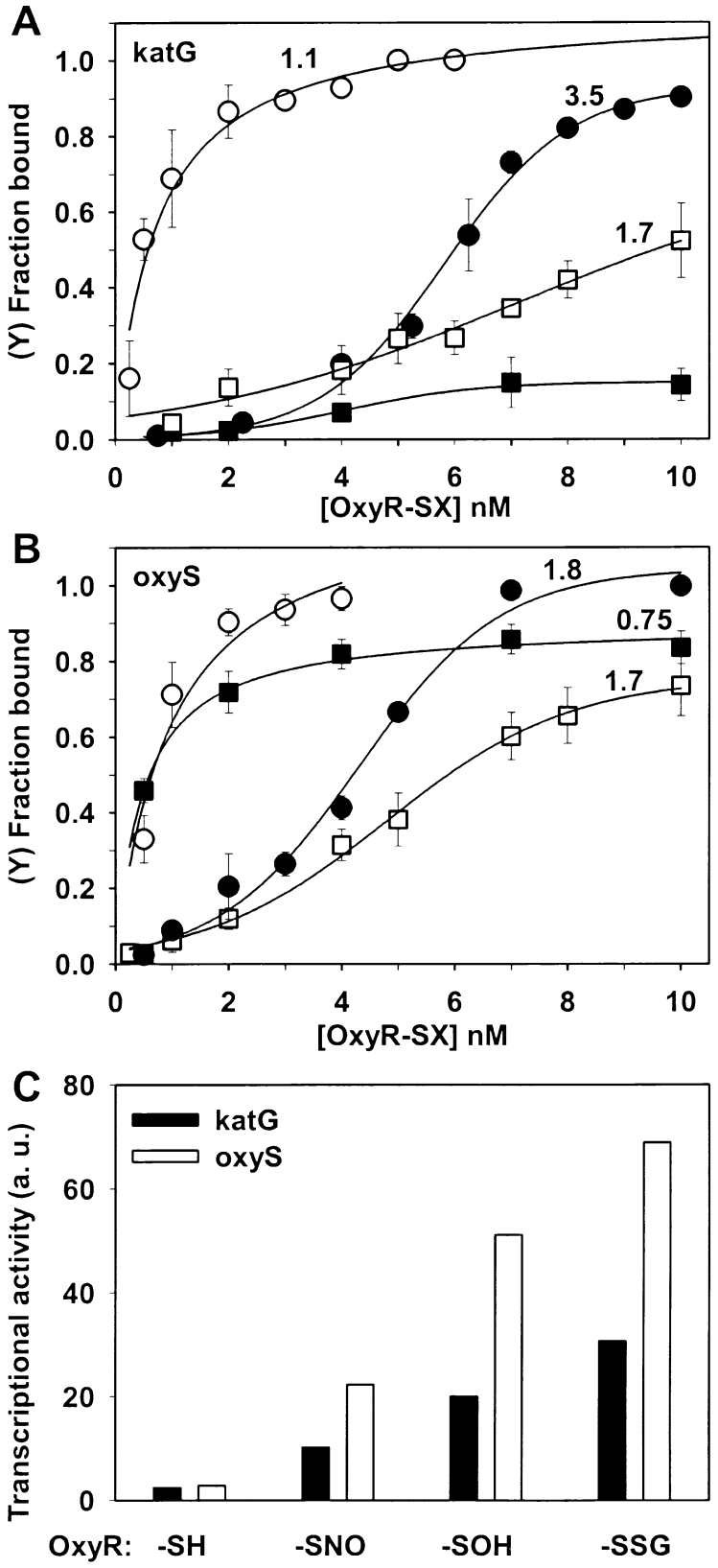


Figure 5. DNA Binding and Activity of OxyR and Its Redox-Related Forms

Fraction of OxyR bound to DNA as a function of OxyR concentration was measured by gel shift assays with DNA fragments containing (A) *katG* binding site and (B) *oxyS* binding site. Binding curves are shown for OxyR-SNO (□), OxyR-SH (■), OxyR-SOH (●), and OxyR-SSG (○). Saturation in OxyR-SNO binding to *kat*G, seen at slightly higher concentrations, was left off the graph for clarity. Hill coefficients (a measure of cooperativity) are provided above each curve. (C) Transcriptional activity of OxyR. Each redox-related form of OxyR (6 nM) was incubated with plasmids containing the *katG* or *oxyS* genes and analyzed by primer extension (n = 2). Band intensities were quantified using a PhosphoImager. a.u., arbitrary units.

but discordant effects on affinity for the *oxyS* and *katG* sites (versus OxyR-SH); and (4) high affinity, noncooperative binding of OxyR-SSG to both promoters. Thus, the mixed disulfide provides the strongest stimulus and shows the least cooperation in response. To examine whether these different binding affinities translate into

different transcriptional potency, we measured the activities of the native reduced and modified forms of OxyR at subsaturating concentrations of activators (6 nM). Figure 5C shows that although the relative potency of the redox-related modifications is the same, both *katG* and *oxyS* genes' (OxyR-SSG > OxyR-SOH > OxyR-SNO) binding affinity does not predict transactivating potency. Thus, the effect of cysteine modifications on binding and activation are separate events. Analysis of mutants showed that the OxyR-SOH derivative of the C208S mutant had partial transcriptional activity. Specifically, the *oxyS* gene was transcribed by C208S to about the same level as the wild-type protein, but *katG* transcription was blunted (but still greater than control OxyR-SH). In contrast, the C199S mutant was transcriptionally inactive (data not shown).

Discussion

Reactive nitrogen and oxygen species are widely used in nature for both physiological signaling and antimicrobial purposes. Proteins that transduce nitric oxide-related signals most often contain a single critical cysteine that is the target of S-nitrosylation, and necessary and sufficient for activity (Hess et al., 2001; Stamler et al., 2001). However, the molecular mechanisms by which oxidative signals are sensed and then transduced into functional changes are less clear because redox-based posttranslational modifications of target proteins have been difficult to produce in pure form. In addition, current models do not directly address how alternative redox signals can be differentially processed to produce distinct cellular responses. Here, we have built highly pure and distinct redox-modified forms of a protein, OxyR. Our studies demonstrate that a single cysteine can be modified to yield a number of stable, transcriptionally active forms, which are distinct in structure as well as in their affinity for DNA and cooperativity of response. These results show that proteins can process different redox-related signals into distinct functional responses, and thus provide insight into how specificity in redox regulation may be achieved.

The Redox-Related Sensor and Switch

Zheng et al. (1998) have reported that redox activation of OxyR occurs via the formation of an intramolecular disulfide bond between C199 (the sensor) and C208. However, the supporting data (biochemical and structural) were obtained with mutant and truncated mutant proteins in which 16 cysteines (4 per subunit) were replaced with alanines (Zheng et al., 1998; Choi et al., 2001). In addition, there are unavoidable caveats with analyses of reactive functional groups that are done on protein digests, and with the use of X-ray crystallography to elucidate the nature of redox modifications: free radicals generated by ionizing radiation, the lengthy crystallization process, and the oxidizing conditions may influence the redox-related product. It is notable that cysteines 199 and 208 are distant (18 Å) in the reduced OxyR protein and that the mechanism of disulfide formation is not readily apparent (Choi et al., 2001). Disulfide formation involves a number of potential reaction intermediates, including S-OH, S-NO, and S-SG. Generation of these species by exposure to RSNO and H_2O_2 should catalyze disulfide formation (Gilbert, 1990; Claiborne et al., 1993; Gitler et al., 1994; Arnelle and Stamler, 1995; Stamler and Hausladen, 1998), but in fact we show that each modification is stable in the wild-type protein and that these alternatively modified proteins have different activities. Such differential responsivity argues against an exclusive or sufficient role for disulfide in the regulatory mechanism. Our studies thus establish that *S*-nitrosylation, *S*-hydroxylation, and *S*-glutathionylation are mechanisms of activation of wild-type OxyR.

Disulfide formation may act as a primary switch in other cases and it remains possible that a preparatory posttranslational modification or an adaptor protein could facilitate the formation of C199-C208 disulfide in vivo and thereby activate OxyR. However, C208 can clearly contribute to the regulatory mechanism of OxyR by other means. One possibility is that C208 plays a role in stabilizing the posttranslational modification of C199, either by chemical means (e.g., formation of an N-hydoxysulfenimide, -SN(O)SR, or "SNO2") or through effects on protein conformation. In addition, we have found a dithiothreitol-accessible disulfide between C180 and C259— which may explain the migration properties of OxyR in gels (Tao, 1999)—and which might influence the regulatory mechanism through allosteric means by analogy to the preparatory role of thiol/disulfide in facilitating *S*-nitrosylation of the ryanodine receptor (Stamler et al., 2001). Collectively, our data suggest that a range of redox-based modifications—S-H, S-S, S-NO, S-OH, and S-SG—may subserve not only direct sensing and switching functions, but may also provide a mechanism for allosteric regulation in redox control (Stamler and Hausladen, 1998; Stamler et al., 2001).

The ability of OxyR to sense multiple redox-related signals is achieved through a remarkable design that includes both the elements of a motif for *S*-nitrosylation as well as a binding site for glutathione disulfide (Pineda-Molina et al., 2001; Stamler et al., 2001; Figure 2). Resident acids and bases surrounding C199 may also increase reactivity toward hydrogen peroxide (Choi et al., 2001). It is likely that sensory mechanisms existing elsewhere will be similarly adapted to recognize specific redox-related molecules.

Redox-Related Structural Changes

The binding affinities of active forms of OxyR correlated with their α helix content: OxyR-SSG > OxyR-SOH > OxyR-SNO—a measure previously associated with improved DNA binding of transcription factors, including members in the redox-sensitive class (e.g., Pax-8 [Tell et al., 1998] and NFκB [Matthews et al., 1995]). However, our data indicate that binding affinities do not generally predict transactivating potencies, which are specific for each redox-related form and promoter. These data are not fully accounted for by the simple concept of a uniform change in DNA conformation produced upon binding a given regulator or by the principle that a regulator may assume distinct conformations upon binding to different genomic sites (Lefstin and Yamamoto, 1998). An additional level of regulation is demonstrated by our results, in which multiple active forms (or states) of a transcription activator can also produce distinct alter-

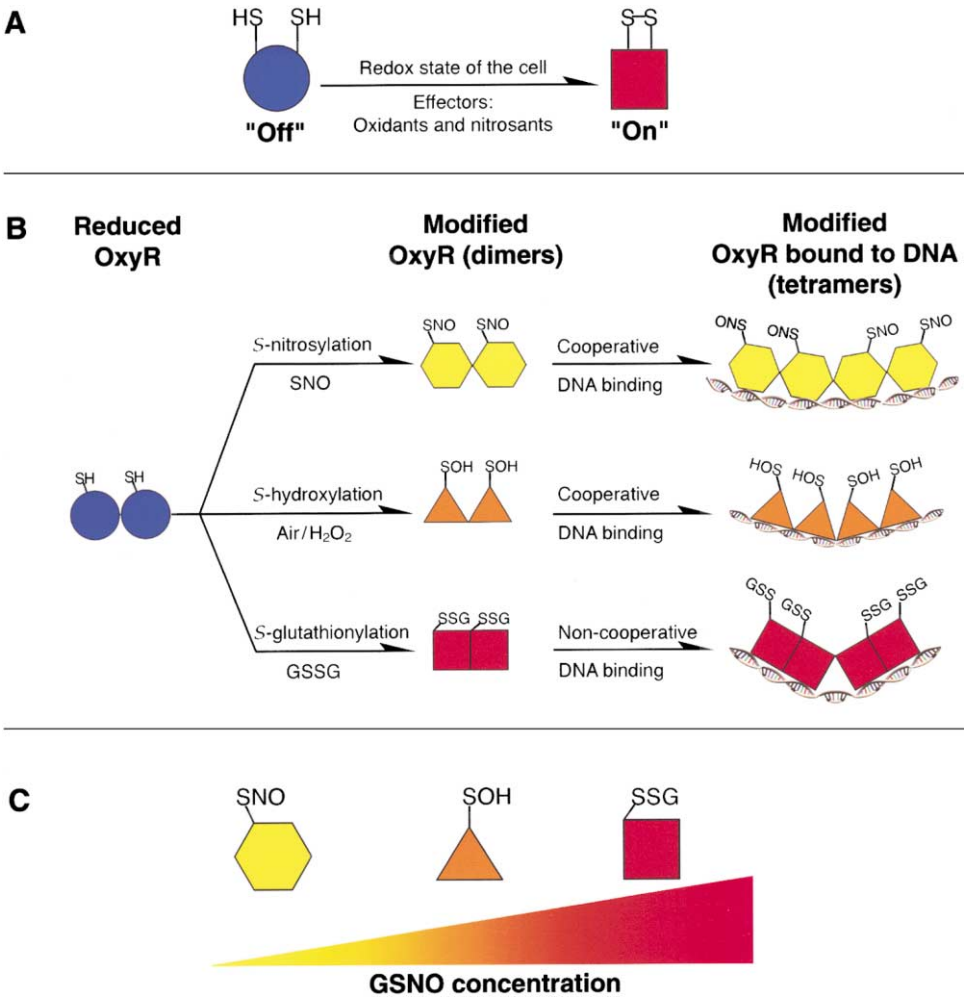


Figure 6. Models for Redox Regulation

(A) The prototypic two-state model. Redox state of the cell determines the on or off status of the protein. All effectors produce the same redox-related modification, prototypically an intramolecular disulfide.

(B) The expanded paradigm for redox-related signaling in which proteins sense and distinguish between different redox-related allosteric effectors by virtue of a redox-based mechanism for cooperativity (exemplified by OxyR binding to promoter sites). *S*-nitrosylation, *S*-hydroxylation, and *S*-glutathionylation produce unique conformational changes in protein subunits, which yield unique DNA conformations and differential responses. Different modifications are generated by varying the redox-related effector (physiological signaling) or by increasing its concentration (nitrosative or oxidative stress) as illustrated in (C). Assignment of the modifications to dimers is based on sedimentation equilibria studies described in Results; however, we do not exclude the possibility that OxyR is modified subsequent to tetramer formation and/or DNA binding, and in fact DNA may serve as an allosteric effector of redox-based modification and of conformational changes governing dimer/tetramer interconversion.

(C) Continuum of redox-related modifications implicated in physiological signaling and cellular stress. A change in concentration of a redox-related effector (eg. GSNO) may result in alternate signals or may serve as the mechanistic basis of oxidative or nitrosative stress. In this model, fundamental differences between redox-related signals and stresses lie not in the modifications themselves but in their consequences for protein function.

ations in the same DNA structure (Figure 4). These findings further raise the possibility of varied motifs or binding sites for OxyR forms with different affinities, and with different consequences for activation of alternative genes.

A Redox Basis for Cooperativity

It has been proposed that transcriptional activators are allosteric proteins akin to enzymes and other regulatory proteins (Lefstin and Yamamoto, 1998; Lewis et al., 1996; Weiss et al., 1992). Allosteric mechanisms have thus been applied to explain cooperative DNA binding of many regulators as well as the control exerted by DNA response elements (i.e., the DNA may be viewed as an allosteric effector; Lefstin and Yamamoto, 1998). Our new data suggest that such mechanisms for cooperative effects in activators can be redox-based. That is, structural variations that alter OxyR affinity and cooperativity of binding to DNA can be rationalized in terms of alternative redox-based modifications of a key cysteine (S-H, S-OH, S-SG, or S-NO) that subserves control of gene expression.

It remains to be determined if cooperativity in OxyR is mediated by changes in the tertiary structure of indi-

vidual subunits that then influence the activity of other subunits, or whether the redox-related modifications govern an equilibrium between quaternary structures with varying affinities for DNA, analogous to the allosteric mechanism for cooperation in hemoglobin (Perutz et al., 1998). In this regard, it would be important to know the effect of cysteine modification on the dimer↔tetramer equilibrium and allosteric transition ("linkage"), and whether the answer is the same for each redox-related modification. It may also be important to know the effects of hybrid OxyR molecules containing, for example, S-NOs on one dimer (at one or both sites) and S-SG or S-OH on the other, as well as the effect of DNA on linked functions.

A Model for Redox-Related Control

Redox regulation of activators has been viewed as a switch with two modes of operation: on or off (Figure 6A). This simple model holds that when cells sense a change in redox state, the redox regulators assume the on position and thereby activate transcription to mount an adaptive cellular stress response (Demple, 1998; Zheng et al., 1998). The model accommodates some possibility for a graded and differential response at the level of molecular recognition by use of sensors that oxidize at different redox potentials and/or otherwise discriminate among redox-related molecules (e.g., H_2O_2 and O_2^-; Hidalgo et al., 1997), although the mechanistic basis for such discriminatory behavior has not been demonstrated.

The new data expand the paradigm of redox regulation to include selective and differential activity in response to different redox-related signals. The model holds that proteins (e.g., transcriptional activators) receiving input from multiple redox-based signaling pathways will be capable of processing the information into distinct functional responses (Figure 6B). This is achieved by protein recognition of a series of redox-related modifications that constitute signaling events, by transcription factors adopting conformational states that are individualized for particular signals, by the cooperative binding of the transcription factors to DNA, and by DNA sites serving as allosteric effectors. In this model, specificity inherent to physiological signaling is provided by alternative modifications that subserve graded and differential responsivity to redox signals (e.g RSNO and H_2O_2; Figure 6B), while oxidative or nitrosative stresses are identified with high affinity, noncooperative binding to DNA (e.g., the consequence of the intracellular increase in GSSG that forms irrespective of the causal agent; Figure 6B and 6C).

Our data indicate that proteins can recognize not only the nature of the redox-related signal or stress, but also its extent, and moreover, that they can process such redox-related information into distinct and graded responses. The evidence for this expanded paradigm of redox regulation derives from a model protein containing a single critical cysteine. The larger potential for diversity in redox-based signaling can be understood by appreciating that proteins may contain as many as 200 free cysteines and multiple molecular classes of redox centers (Eu et al., 2000; Sun et al., 2001).

Experimental Procedures

Chemical Characterization and Quantitation of Redox-Related Modifications

Thiol in OxyR was measured using the monobromobimane fluorescence based assay (Kosower and Kosower, 1987; Xu et al., 1998), with GSH as the standard (range from 25–0.78 μM), and was confirmed with the Saville method (Saville, 1958). Additional thiol quantification and identification was done by amino acid analysis and mass spectroscopy using iodoacetamide labeling (see below).

The OxyR-SOH modification was quantified by reduction of the air-oxidized form of OxyR with the SOH-specific reductant sodium arsenite (10 mM; Radi et al., 1991). The identity of this modification was further confirmed by the dimedone assay (DeMaster et al., 1995), and by the spectral shift produced by reaction of sulfenate with NBD (Ellis and Poole, 1997). Specifically, 400 μM NBD was reacted with either 10–15 μM air oxidized OxyR or anaerobically reduced OxyR (in 25 mM potassium phosphate buffer with 0.3 M KCl and 100 μM diethylenetriaminepentaacetic acid [DTPA] at pH 8), for 1 hr at 25°C. Spectra were recorded in a Cintra 40, UV-Vis spectrometer (GBC Scientific) from 250 to 700 nm against a blank without protein, using 0.1 cm pathlength quartz cuvettes (Hellma, Inc.). Sulfenate derivatization by NBD was also assayed by mass spectroscopy (see below).

The reduced form of OxyR (OxyR-SH) was generated by the addition of a large excess of DTT (180–200 mM) for 1 hr, followed by exhaustive dialysis (25 mM potassium phosphate, 250 mM potassium sulfate, 1 mM magnesium sulfate, and 100 μM DTPA [pH 8]) in an anaerobic glove box (Coy Laboratory Products Inc.). Removal of DTT was monitored by the color change in the dialysate after addition of 5,5′-dithionitrobenzoic acid to an aliquot of the dialysis buffer. In some experiments, 500 μl aliquots of argon-degassed protein were reduced by exposure to sodium arsenite or sodium cyanoborohydride (10 mM) for 1 hr.

OxyR-SNO was generated by adding a 10- to 50-fold excess of S-nitrosocysteine or S-nitrosoglutathione (preferred) to OxyR-SH under anaerobic conditions for 1–2 hr (see above), followed by dialysis. Complete removal of excess CSNO or GSNO and the stoichiometry of S-nitrosylated OxyR were determined by photolysis/chemiluminescence or the modified method of Saville (Stamler and Feelisch, 1996) as previously described (Hausladen et al., 1996). (Exposure of OxyR-SH to >50-fold excess GSNO for long periods to simulate high levels of nitrosative stress generated variable amounts of glutathione mixed disulfide).

OxyR-SSG was synthesized by exposure of OxyR-SH to 10-fold excess GSSG for 2–3 hr under strictly anaerobic conditions followed by extensive dialysis. Quantification was done by HPLC. Specifically, 5 mM DTT was added to a 100 μl aliquot of OxyR-SSG for 30 min in order to release the GSH from the mixed disulfide. Following ultrafiltration of protein (Amicon Inc.), monobromobimane (10 mM) was added (for 30 min). The GS-MBBr complex was then separated by HPLC using a C_{18} column, 0%–10% gradient from sodium acetate (pH 6.8) to acetonitrile. Detection was done by fluorescence (Ex = 382 nm; Em = 482 nm) and the quantification of mixed disulfide was derived from a GS-MBBr standard curve. OxyR-SSG was also assayed by mass spectroscopy (see below).

ESI/MS

Electrospray ionization (ESI) experiments were performed on a Micromass Q-Tof ™ II (Micromass, Wythenshawe, UK) mass spectrometer equipped with an orthogonal electrospray source (Z-spray) operated in positive ion mode. Sodium iodide was used for mass calibration for a calibration range of m/z 100–2500. Proteins were prepared in a solution containing 50% acetonitrile/50% water, 0.1% formic acid at a concentration of ~50 pmol/μl and infused into the electrospray source at a rate of 5–10 μl min^{-1}. Optimal ESI conditions were: capillary voltage 3000 V, source temperature 110°C, and a cone voltage of 60 V. The ESI gas was nitrogen. Q1 was set to optimally pass ions from m/z 500–2000 and all ions transmitted into the pusher region of the TOF analyzer were scanned over m/z 500–3000 with a 1 s integration time. Data was acquired in continuum mode until acceptable averaged data was obtained (10–15 min). ESI data was deconvoluted using MaxEnt I provided by Micromass.

MALDI-TOF/MS

Trypsin (2 μl of 1 mg/ml) was added to IA and NBD modified OxyR (500 μl of 150 μg/ml) and the mixture was incubated for 5 hr at 37°C. Subsequently, 5 μl of 10% trifluoroacetic acid (TFA) was added to stop the digest. Alternatively, TLCK (1-chloro-3-tosylamido-7-amino-2-heptanone-HCl) treated chymotrypsin (2 μl of 2 mg/ml) was used. MALDI-TOF was performed on a Bruker Reflex III (Bruker, Bremen, Germany) mass spectrometer operated in linear, positive ion mode with an N_2 laser. Laser power was used at the threshold level required to generate a signal. Accelerating voltage was set to 28 kV. The instrument was calibrated with protein/peptide standards bracketing the molecular weights of the protein/peptide samples (typically mixtures of apo-myoglobin and bovine serum albumin using doubly charged, singly charged, and dimer peaks as appropriate or bradykinin fragment 1-5 and adrenocorticotropic hormone fragment 18-39 for tryptic digest analysis). Samples were prepared in 0.1% TFA at an approximate concentration of 50 pmol/μl. Sinapinic acid was used as the matrix for proteins and α-cyano-4-hydroxycinnamic acid for peptides prepared as saturated solutions in 50% ACN/ 0.1% TFA (in water). Allotments of 1 μl of matrix and 1 μl of sample were thoroughly mixed together; 0.5 μl of this was spotted on the target plate and allowed to dry.

Amino Acid Analysis

Carboxymethylation

A concentrated stock protein solution was diluted approximately 10-fold, yielding a final protein concentration of 0.5 mg/ml in 100 mM Tris/HCl buffer (pH 8.0) containing 1 mM EDTA and supplemented with either 5 mM DTT or 5 mM DTT + 6 M guanidine HCl. A control reaction, containing buffer only, was also prepared. Following a 120 min incubation at 37°C, iodoacetamide (200 mM stock solution prepared in water) was added at either 10 mM or, in the case of DTT pretreatment, 2-fold molar excess of total thiol concentration. The samples were further incubated at 37°C for an additional 1–2 hr followed by dialysis (in the dark) against several changes (1/1000; volume sample/volume dialysis buffer) of the indicated buffer.

Analysis

Samples were dialyzed (using tubing; Gibco/Life Technologies; with a 10,000 molecular weight cutoff) exhaustively against 5 mM NH_4HCO_3 (pH 8.5). Measured aliquots (typically 5–200 μg in 50–250 μl) were delivered into 1.0 ml prescored glass ampoules (Wheaton, previously pyrolyzed at 350°C for 48 hr). The ampoules were frozen, lyophilized to dryness, reconstituted with 100 μl of HPLC grade H_20, frozen, and relyophilized. This process was repeated a total of three times. 100 μl of hydrolysis reagent (6 N HCl supplemented with 0.1% phenol and 0.04% 2-mercaptoethanol) was then added and the vials were evacuated with a vacuum pump and flushed with nitrogen (repeated for a total of three cycles). The ampoules were evacuated and finally flame sealed. Hydrolysis of the protein was performed by placing the sealed ampoules in a sand bath present in an oven maintained at 150 ± 2°C for 60 min (Hare, 1977). The hydrolysis reagent was removed under reduced pressure and samples were reconstituted with 100 μl of NaS (Beckman Coulter, Fullerton, CA) buffer diluent. Appropriate sample dilutions were made with additional NaS buffer to yield approximately 0.5–1 nmol per 50 μl injection. A Beckman 6300 High Performance Amino Acid Analyzer interfaced to an IBM computer equipped with Beckman 32 K chromatography software for data collection and analysis provided the efficient chromatographic resolution of all amino acids when equipped with a short column and 3 buffer sodium citrate system (NaA, NaB, and NaD) provided by the manufacturer. Gradient profiles were run according to manufacturer's recommendations. Data was corrected for CM-Cys destruction, previously determined to be ~12%–15% for the indicated hydrolysis conditions. An internal standard (norleucine) was added to correct for recovery and destruction of labile amino acids.

Preparation of Samples for CD

CD spectra of OxyR were obtained using 1 ml aliquots of the purified protein (0.4–0.7 mg/ml), dialyzed into CD buffer (25 mM potassium phosphate, 100 mM potassium sulfate, 1 mM magnesium sulfate, and 100 μM DTPA, [pH 8]), which has minimal absorbance in the low UV region. Sodium cyanoborohydride (10 mM) proved to be the preferred reducing agent for the study of OxyR-SH, because it does not absorb in the far UV region, and therefore obviated the need for a dialysis step in the protocol. OxyR-SNO CD was obtained both aerobically and anaerobically.

Samples were placed in a CD quality quartz cuvette (0.1 cm path length), with a Teflon stopper. 5–10 individual spectra were accumulated and averaged to create a data set. An AVIV spectrometer was used to collect the spectra at a wavelength range of 185–260 nm, with a scan speed of 30 nm/min, and a photomultiplier sensitivity range of ±10 mdeg at 25°C. Spectra were analyzed using "Selcon," a self-consistency algorithm (Sreerama and Woody, 1993).

Spectra of the protein-DNA complexes were generally handled in the same way as the isolated proteins. The DNA oligonucleotides were added to the protein samples inside the anaerobic chamber and sealed in the cuvettes before they were transferred to the CD spectrometer.

Protein and DNA Purification and Concentration

OxyR was purified, prepared, and stored as described previously (Hausladen et al., 1996). Protein concentration was determined using the Coomassie assay (Pierce Chemical Co.) with BSA as a standard, a method previously verified by amino acid analysis (Hausladen et al., 1996). The DNA used for CD analysis was a 48 bp synthetic oligonucleotide fragment of the *katG* promoter (Toledano et al., 1994). The oligonucleotides were synthesized and purified by gel electrophoresis in the biosynthesis division at Duke or obtained from a commercial source (Gibco). DNA concentration was determined from the absorbance at 260 nm.

In Vitro Transcription and Primer Extension

In vitro transcription with the various redox-related forms of OxyR was performed as previously described (Hausladen et al., 1996). Plasmid pBT22 was used as the *katG* template and pUCOXYS, a plasmid containing the *oxyS*-coding region cloned into pUC19, was used as the *oxyS* template. Where indicated, redox-related forms of OxyR were reduced with either 200 mM DTT or 10 mM sodium arsenite. Primer extension assays were performed using AMV reverse transcriptase (Promega, Inc.), according to instructions supplied by the manufacturer.

DNA Binding Assays and Cooperativity

Gel mobility shift assays were carried out as described (Ausubel et al., 2000), using conditions in Tartaglia et al. (1992). Freshly modified OxyR was diluted immediately before each binding reaction. Binding reactions were carried out in a final volume of 25 μl and contained the indicated amount of OxyR protein, 3×10^{-12} M probe (106 bp of *katG* DNA or 101 bp of *oxyS* DNA), 25 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 10% glycerol, 0.5 mM EDTA, 0.05% Nonidet P-40, 50 mM NaCl, and 2 ng/μl poly dI.dC. The reactions were incubated at 25°C for 30 min. All samples were loaded on 4% polyacrylamide gel (19:1 acrylamide:bis ratio) and electrophoresed in Tris-acetate buffer at 100 V. Quantitation was done with a PDI Quantity One software package. Binding data was fitted to the Hill equation $1/Y = 1 + (x/x_{50})^{-n}$ where Y is the fraction of DNA bound at OxyR concentration x, x_{50} is the OxyR concentration at 50% saturation, and n is the Hill coefficient.

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