

Nitrosation and oxidation in the regulation of gene expression

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ABSTRACT A growing body of evidence suggests that the cellular response to oxidative and nitrosative stress is primarily regulated at the level of transcription. Posttranslational modification of transcription factors may provide a mechanism by which cells sense these redox changes. In bacteria, for example, OxyR senses redox-related changes via oxidation or nitrosylation of a free thiol in the DNA binding region. This mode of regulation may serve as a paradigm for redox-sensing by eukaryotic transcription factors as most—including NF- κ B, AP-1, and p53—contain reactive thiols in their DNA binding regions, the modification of which alters binding *in vitro*. Several of these transcription factors have been found to be sensitive to both reactive oxygen species and nitric oxide-related species *in vivo*. It remains entirely unclear, however, if oxidation or nitrosylation of eukaryotic transcription factors is an important mode of regulation, or whether transcriptional activating pathways are principally controlled at other redox-sensitive levels.—Marshall, H. E., Merchant, K., Stamler, J. S. Nitrosation and oxidation in the regulation of gene expression. *FASEB J.* 14, 1889–1900 (2000)

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OXIDATIVE STRESS

OXIDATIVE STRESS PLAYS a role in the pathogenesis of many degenerative diseases. The cellular responses to oxidative stress are important for survival of the organism at large and for the normal function of the respiratory system in particular. Adaptations include the induction of the antioxidant enzymes catalase, superoxide dismutase (SOD), and γ -glutamyl cysteine synthetase (γ -GCS) and prompt proteolytic degradation of oxidatively damaged proteins (1, 2). The redox-sensing mechanisms underlying these cellular defenses are at least partly controlled at the level of transcription. However, little is known of the molecular basis of such redox regulation in eukaryotic cells.

Antioxidant defenses are characterized best in

Escherichia coli, where the transcription factors OxyR and SoxRS induce protective genes in response to hydrogen peroxide and superoxide, respectively. The redox signal is sensed by an [2Fe-2S] cluster in SoxR (3) and a free cysteine residue in OxyR (*vide infra*) (4). It is speculated that transcription factors in eukaryotes use similar molecular mechanisms to orchestrate the antioxidative response, but there are no well-elucidated examples. The limitations of the field are highlighted by an appreciation that the purported oxidative modifications of transcription factors have not been produced in pure form nor have any been identified *in vivo*.

NITROSATIVE STRESS

It has been clearly demonstrated that nitric oxide (NO) or related molecules can covalently modify cysteine residues in proteins (5) and that such posttranslational modification can serve in the regulation of cellular responses (5–11). In the strictest sense, S-nitrosylation is the only redox-related modification of proteins that has met the criteria of a physiological signal, that is: 1) the covalent modification has been shown to occur *in vivo*; 2) it alters protein function; and 3) changes in signal amplitude (S-nitrosylation/denitrosylation) have been documented over the time scales of the physiological responses (10, 11). The concept of nitrosative stress has emerged from an understanding that nitrosylation can also reach a hazardous level. Under such conditions, nitrosylation may directly inhibit critical protein functions (12, 13) and/or promote deleterious oxidative modifications that do so (14). At the cellular level, nitrosative stress has been linked to inhibition of cell growth and apoptosis, and thus may be widely implicated in NO pathogenesis. The response has been best studied in *E. coli*, which up-regulate specific anti-nitrosative defenses, including enzymes that metabolize NO and S-nitrosothiols

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(SNOs) (15, 16). In particular, genes under the control of OxyR encode for proteins that metabolize nitrosants and confer a survival advantage for bacteria (17). In other words, OxyR has evolved such that it senses both oxidative and nitrosative events, and responds by up-regulating genes that afford protection from both types of threat. It is of great importance that the S-nitrosothiol derivative of OxyR has been identified *in vivo* (17), as this remains the only redox-related modification of a transcription factor that has been shown to occur within a cellular context.

In the inflammatory response, many of the same cells that generate reactive oxygen species (ROS) also express NO synthases. In some cases, interactions between nitrosants and oxidants may produce products that are more toxic than either reactant alone. In other instances, nitrosative mechanisms of cellular injury predominate (13, 14). One might therefore speculate that eukaryotic cells would have evolved transcriptional control mechanisms analogous to bacteria that sense and respond to both oxidative and nitrosative stress. In this scenario, NO- and O₂-related modifications of proteins would likely constitute biological signaling events in cellular defense mechanisms (e.g., cytokine stimulation). Here we review the response of a number of transcription factors to oxidative and nitrosative stresses. In our attempt to elucidate the molecular events that constitute biological signals, we draw correlates between prokaryotic and eukaryotic transcription factors.

MOLECULAR RECOGNITION

SoxR

Recent headway has been made in elucidating the mechanisms of redox sensing by prokaryotic transcription factors. The redox iron center in SoxR is arranged as a pair of [2Fe-2S] clusters, one in each subunit of the homodimer. The iron is not required to maintain folding of the polypeptide or to mediate protein–DNA interaction. Rather, the iron somehow alters the structure of the promoter complex to initiate transcription by RNA polymerase. How is SoxR activated? Elegant genetic and biochemical studies suggest that oxidation of the iron serves as the functional switch that controls gene expression (18). Nevertheless, it has been difficult to ascribe this event to relevant oxidants *in vivo*. That is, superoxide can oxidize SoxR but does not achieve a cellular steady-state concentration high enough to activate transcription. In contrast, NO cannot oxidize SoxR, but clearly regulates protein activity *in vivo* (15). Thus, it has been alternatively proposed that SoxR responds to a change in the redox state of the cell

(between –260 and –280 mV). Specifically, the redox potential of the [2Fe-2S] cluster is approximately –280 mV (18), which would make it responsive to NADPH/NADP⁺ ratios at the cellular level (19). A remaining conundrum, however, is that SoxR is not activated by H₂O₂, which can both oxidize the cluster and raise the redox potential of the cell above –285 mV. The nature of the redox sensor notwithstanding, SoxR has no known eukaryotic homologue, and none of the eukaryotic transcription factors identified to date contains a redox-active transition metal.

OxyR

The OxyR protein is a homotetramer that is activated by both hydrogen peroxide and S-nitrosothiols (SNOs) (17). The protein contains six cysteines, one of which is absolutely essential for activity and two that are required for maximal activation (4). The protein does not contain a redox-active transition metal. It is unclear from available data what the molecular basis is for OxyR activation. Recent studies with a mutant protein (in which 4 of 6 cysteines per monomer were replaced with alanines) suggested that oxidation of a single thiol to a sulfenic acid may represent the sensor mechanism, whereas the activation mechanism was ascribed to formation of an intramolecular disulfide (S-S) (4, 20). These data are, however, difficult to reconcile with the observation that a stable SNO can form in wild-type OxyR, because S-nitrosylation should have catalyzed disulfide formation with release of the NO group (14, 21); therefore, the data do not rule out the possibility of alternative mechanisms of activation *in vivo*. Indeed, the redox potential of OxyR is approximately –185 mV, making the protein susceptible to thiol/disulfide exchange (20). Accordingly, S-thiolation with glutathione, for example, may serve as a regulatory posttranslational modification in cells. Although there are no known eukaryotic homologues of OxyR, the bacterial OxyR binding motif has been shown to function as a redox-dependent transcriptional enhancer in murine cells (22).

Activation of OxyR by SNOs has been attributed to S-nitrosylation of a single cysteine residue. Components of an S-nitrosylation motif, X (H, R, K) C (D, E), surround Cys 199 in OxyR, making it a likely candidate site of posttranslational modification (23, 24). However, these data do not definitively exclude involvement of a second thiol or an oxidative mechanism of activation because S-nitrosylation can promote oxidative chemistry (17), and broader usage of the S-nitrosylation motif to include additional redox modifications has been considered (23). In one scenario, the molecular modification may be dictated by the identity of the nitrosant or by the

protein structure. That being said, the stability of the S-NO bond in nitrosylated OxyR argues against further oxidation of the critical thiol (Cys 199) by SNOs. It is therefore tempting to speculate that the second conserved thiol in OxyR (Cys 208) plays a role in stabilization of the S-NO bond (as in formation of an N-hydroxysulfenamide) analogous to complexes formed from sulfenic acids (20, 25). More generally, redox responsiveness in proteins may be conferred by S-NO, S-OH, S-S (intramolecular disulfide), and S-SR (mixed disulfide), all potential reversible modifications of reactive cysteines (14) (Table 1). Most redox-responsive transcriptional activators in mammalian cells are likely to be regulated by one of these modifications (i.e., they possess cysteines subject to redox control) and to contain an S-nitrosylation/redox motif (or at least critical components of it) (23). It is as yet unknown, however, whether these oxidative modifications occur *in vivo* or if proteins can process these different signaling modifications into distinct functional responses.

NF-κB

The prototypic form of the redox-sensitive transcription factor NF-κB is the p50-p65 heterodimer, but other hetero- and homodimeric species are found. In its inactive state, NF-κB (p50-p65) is bound to an inhibitory protein, IκBα, and is sequestered in the cytoplasm. With an appropriate stimulus, NF-κB is released from IκBα and translocates to the nucleus, where it can activate target gene transcription (26). NF-κB was initially described in lymphoid cells, but its immunomodulatory role has been clearly established in macrophages, respiratory epithelial cells, endothelial cells, and smooth muscle cells (27–30). NF-κB regulates a wide array of genes involved in the inflammatory response including the cytokines tumor necrosis factor α (TNF-α), interleukin 2, and interleukin 8; adhesion molecules ICAM-1 and VCAM-1; and nitric oxide synthase 2 (NOS2) (31).

Initial studies were unclear as to whether an oxidizing or reducing environment favored NF-κB activation (32, 33). It now seems more certain that reducing conditions are required in the nucleus for NF-κB DNA binding (34–36), whereas oxidizing

conditions in the cytoplasm promote NF-κB activation (37–39). Pretreatment of cells with the antioxidants N-acetyl-L-cysteine or pyrrolidine dithiocarbamate (PDTC) prevents cytokine-induced NF-κB activation (32, 38, 39). Furthermore, hydrogen peroxide (H₂O₂) and superoxide (O₂^{•−}) activate NF-κB in certain cell systems (38, 40, 41). In the cytosol, mitochondrial ROS may mediate the changes in cytoplasmic redox state that signal NF-κB activation. Specifically, blocking production of oxygen radicals from the electron transport chain prevents the activation of NF-κB in cytokine-stimulated cells (42); mitochondrial MnSOD expression is increased by NF-κB, perhaps in anticipation of redox signaling (29). However, the redox-sensitive step or ‘on switch’ for NF-κB in the cytoplasm is still not known. One potential target is p21 ras, which is activated by oxidative stress leading to an increase in NF-κB activity (43). The activation of NF-κB by ras is not dependent on nuclear translocation of p50-p65, but rather ras initiates a MAP kinase cascade that results in the phosphorylation of the p65 subunit (44, 45). Phosphorylation of p65 then augments NF-κB-dependent gene transcription (46).

Once NF-κB is released from the IκB regulatory protein, it rapidly translocates to the nucleus. However, for NF-κB to bind to DNA, a cysteine residue in the DNA binding region of the p50 subunit (cysteine 62) must be in a reduced state (36, 47). This cysteine has been shown to participate in intermolecular disulfide formation (34). Moreover, thioredoxin, a protein that reduces disulfides, and redox factor 1 (Ref-1), a reducing molecule unique to the nucleus, function to regulate the redox status of NF-κB in the nucleus (35). Recently it has been shown that thioredoxin translocates to the nucleus concomitant with NF-κB activation and may physically interact with the p50 subunit prior to DNA binding (48).

Taken together, these data make an excellent case for the redox regulation of NF-κB responses. However, for all of these data there is no firm evidence that NF-κB proteins undergo oxidative posttranslational modifications *in situ*. In other words, the particular sites within NF-κB that might undergo redox modification (i.e., p50, p65, IκB proteins, IκB kinases) in cells have not been clearly identified and the nature of the posttranslational modifications that serve in these cellular control mechanisms are not known (Fig. 1A).

NO is the prototypic redox-signaling molecule—more versatile than O₂^{•−} or H₂O₂ and clearly better identified with redox-related modifications of intracellular proteins (6, 14). NO and NF-κB signaling pathways are intimately linked. NF-κB activation is essential for NOS2 gene transcription (49), and NO-related molecules modulate NF-κB signal transduction in a cell- and stimulus-specific manner. For

TABLE 1. Reversible thiol modifications induced by reactive nitrogen species^a (RNS) and reactive oxygen species (ROS)

	S(NO) _x	S-OH	S-SR	S-S
RNS	+	+	+	+
ROS	—	+	+	+

^a Reactive nitrogen species in this context refers to nitric oxide or related molecules. See ref 12 for details of RNS/ROS identity. X = 1 or 2 (little is known about the case of x = 2; refer to refs 142, 143 for examples). See text for details.

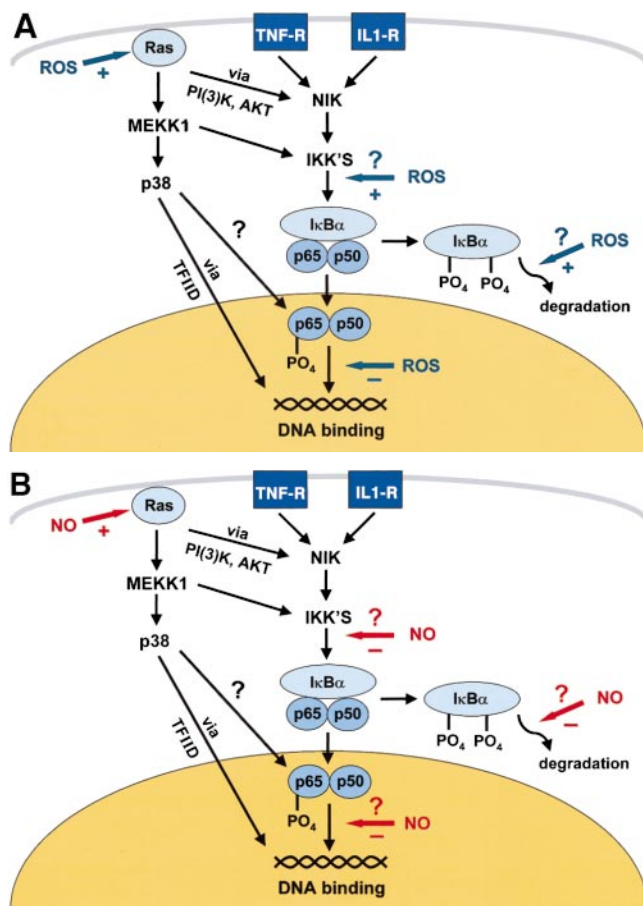


Figure 1. Diagram of the NF-κB activation pathway indicating the steps where there is evidence for direct modulation by A) reactive oxygen species (ROS) or B) NO or related molecules (NO). In the case of NO, S-nitrosylation is the most likely posttranslational modification. The identities of the ROS-related modifications are not known, however. See text for further details. IKK, IκB kinase; NIK, NF-κB-inducing kinase; PI(3)K, phosphatidylinositol 3-kinase; TFIID, transcription factor IID.

example, low concentrations (0.1 to 10 μM) of NO, or related donors, activate NF-κB in human lymphocytes (50). Here, the mechanism is indirect: S-nitrosylation of a conserved cysteine residue in the guanine binding domain of p21 ras leads to NF-κB activation (8). Epstein-Barr virus-infected lympho-

cytes may take advantage of this pathway to selectively control the expression of a virus-specific transcriptional activator termed Zta (51). Higher concentrations of NO species (0.2 to 0.5 mM), on the other hand, inhibit NF-κB activation in hepatocytes (52), T cells (53), endothelial (27), and vascular smooth muscle cells (28) and prevent DNA binding of both the NF-κB p50-p65 heterodimer and p50 and p65 homodimers *in vitro* (54). Conversely, inhibition of endogenous production of NO-related activity augments NF-κB activity in macrophages and endothelial cells (27, 55).

NO or related molecules appear to inhibit NF-κB activation through a multiplicity of mechanisms. Studies in endothelial cells show that nitrosothiols prevent the proteolytic degradation of the IκBα complex in the cytoplasm by increasing transcription of I-κBα and/or stabilizing the mRNA transcripts (27). But other work *in vitro* indicates that nitrosylation of the cysteine 62 residue in the p50 subunit prevents NF-κB from binding to DNA and activating transcription, and we have recently detected S-nitrosylated NF-κB in cells (unpublished observations). The physiological significance of this posttranslational modification is, however, less clear (54). A summary of the available data suggests that NO affects NF-κB differently depending on the cell type, activating stimulus, NO-related species, NO concentration, and redox state of the cell (Fig. 1B and Table 2). This is nicely exemplified in murine macrophages, where a biphasic response to NO-related molecules is seen for NF-κB-dependent expression of NOS2 (56).

AP-1, c-Jun, and c-Fos

AP-1 is a transcription factor that belongs to the basic leucine zipper (bZip) family. It is a heterodimeric protein consisting of c-Fos and a Jun (c-Jun, JunB, JunD) subunit, with the primary activating form being c-Fos/c-Jun. Jun-Jun homodimers also form, however, and can activate transcription, albeit less efficiently than the Fos/Jun heterodimer (57). Other proteins of the Fos family (i.e., FosB, Fra1,

TABLE 2. Effects of NO-related molecules on NF-κB^a

Cell type	Source of NO	Concentration	Stimulus	Effect	Proposed mechanism
Human lymphocytes	SNAP	0.1–10 μM	none	↑ Activation	S-nitrosylation of p21 ras
Human endothelial cells	SNP	1 mM	TNFα	↓ Activation	↓ degradation and ↑ transcription of IκBα
Human smooth muscle cells	GSNO	0.2 mM	IFN-γ and IL-1α	↓ Activation	None proposed
	NOR3 or SNP	0.01–1 mM	IL-1β	↓ Activation	↓ phosphorylation and degradation of IκBα
p50 and p50-p65 dimers <i>in vitro</i>	SNP	1–5 mM	N.A.	↓ DNA binding	S-nitrosylation of a thiol on the p50 subunit
	SNAP	0.01–1 mM			

^a Demonstrating cell, concentration, and stimulus specificity.

Fra2) can form dimers with Jun proteins and bind to the AP-1 site, but do not have trans-activation potential (58). AP-1 activation has an important role in the control of cell proliferation and differentiation. Several different growth factors (e.g., bFGF, EGF), cytokines (e.g., TNF- α , TGF β), and tumor promoters (e.g., phorbol esters, asbestos fibers) increase cellular AP-1 activity *in vitro* (57, 59, 60).

Classical regulation of cellular AP-1 activity occurs through two mechanisms: an increase in the transcription of c-fos and c-jun, and the phosphorylation of c-Fos and c-Jun proteins. The serum response element and TPA response element regulate the transcription of c-fos and c-jun respectively (61). JNK1/2 (c-Jun NH₂-terminal kinase), ERK1/2 (extracellular stimulus responsive kinase), and other members of the MAP kinase family, participate in the phosphorylation of the c-Jun and c-Fos proteins (61–63).

The existing data on the redox regulation of AP-1 are quite confusing. Antioxidants have been shown to increase cellular AP-1 activity (i.e., the reverse of what is seen with NF- κ B) and AP-1 activity is highest in cells that have the highest levels of thioredoxin (37, 64). However, many of the studies that show activation of AP-1 and JNK by antioxidants were performed using PDTC and butylated hydroxyanisole. While these compounds do have antioxidant properties, they can also induce oxidative stress in cellular systems (65) and/or modify protein thiols directly. In addition, the activation of AP-1 by these compounds is attenuated by an increase in cellular thiol levels (65). Thus, the precept that AP-1 is activated by antioxidants needs to be reexamined.

Indeed, the evidence seems stronger that oxidative stress induces AP-1 transcriptional activity. Hydrogen peroxide and superoxide-generating systems increase the transcription of c-fos and c-jun, which leads to an increase in AP-1 activity (66–68). The stimulation of AP-1 activity by cytokines and growth factors also appears to be dependent on superoxide production (69, 70). Moreover, increased cellular glutathione levels inhibit the expression of c-Fos and Jun proteins as well as the activation of AP-1 by numerous stimuli (65, 70–73). This redox sensitivity is likely transduced through the JNK and ERK pathways (70). In conclusion, review of the available data to date indicates that AP-1 is not antioxidant responsive, but rather is activated by either oxidants or oxidative stress.

Just as there is debate over whether oxidants or antioxidants activate AP-1, there is some uncertainty as to whether AP-1 trans-activates the antioxidant response element (ARE). The ARE has been found in several antioxidant genes including NAD(P)H:quinone reductase, glutathione S-transferase, and γ -GCS (74, 75). Although AP-1 or AP-1 like elements

exist in the ARE, AP-1 dimers containing c-Fos or Fra1 subunits actually inhibit transcription (76) and the consensus is that AP-1 is not the primary trans-activating protein (76–78). Rather, two other bZip transcription factors, Nrf1 and Nrf2, are viewed as the primary regulators of the ARE (76, 79–81).

Much like NF-kappa B, AP-1 binding to DNA is favored under reducing conditions. A single cysteine residue in the basic region (i.e., DNA binding domain) of the c-Fos and c-Jun proteins confers this redox sensitivity (82). Ref-1 appears to be the principal regulator of the redox status of AP-1 (83). Ref-1, in turn, is regulated by thioredoxin, which translocates to the nucleus on cell stimulation and interacts with Ref-1 purportedly through the formation of a disulfide bond (84). Deletion of a single cysteine residue in the thioredoxin molecule prevents this interaction, thereby decreasing AP-1 activation. The reducing capability of Ref-1 is further dependent on a cysteine residue located at the NH₂-terminal end of the protein (85). Of note, Ref-1 also has DNA repair activity that is located in a different domain of the protein (86). None of these data elucidate the molecular basis of the redox sensitivity of AP-1 as it relates to DNA binding.

Recent studies with c-Jun *in vitro* demonstrate that a decrease in the GSH:GSSG ratio induces S-glutathiolation of the redox-sensitive cysteine in the basic region and prevents c-Jun DNA binding (87). Oxidative modification of a single cysteine is therefore key to modulation of its activity *in vitro*. In principle, a sulfenic acid, a related condensation, intramolecular and intermolecular disulfides, or combinations of the above are all potential posttranslational modifications of AP-1 that may serve as part of a transcriptional control mechanism.

Cellular treatment with NO donor compounds inhibits AP-1 binding to DNA (88). Additional *in vitro* studies show that NO or related molecules inhibit c-Jun and c-Jun/c-Fos DNA binding in a reversible manner (89). This inhibition is mediated via reactions with conserved cysteines in the DNA binding region of both c-Jun and c-Fos and is best rationalized by S-nitrosylation. Recent experiments showing that high NO donor concentrations can induce S-glutathiolation of the critical cysteine in c-Jun make it clear, however, that oxidation of this residue can not be definitively excluded (90). NO effects on AP-1 activity in biological systems are moreover cell type specific and concentration dependent (**Table 3**). In one system, NO-related compounds actually increased AP-1 binding and transcription of c-fos and c-jun (91–93). This activity may have been secondary to stimulation of guanylate cyclase as comparable degrees of AP-1 activation were elicited with cGMP analogs (92, 94); the authors also implicated a cGMP-independent pathway

TABLE 3. Effects of NO-related molecules on AP-1^a

Cell or tissue type	Source of NO	Conc.	Stimulus	Effect	Proposed mechanism
Nuclear extracts of mouse cerebellar granule cells and fibroblasts	SNP	1–5 mM	none	↓ AP-1 binding	S-nitrosylation of a thiol on AP-1
Rat embryo fibroblasts	SNP	0.1 mM	none	↑ AP-1 activation	↑ transcription of jun B and c-fos
Mouse cerebellar granule cells	GSNO	0.05–0.2 mM	NMDA	↓ AP-1 activation	↓ c-fos transcription
c-Fos and c-Jun dimers <i>in vitro</i>	SNP	0.05–0.2 mM	TPA	↓ DNA binding	S-nitrosylation of a thiol on c-Jun and c-Fos
c-Jun homodimers <i>in vitro</i>	NO	0.2–0.8 mM	N.A.	↓ DNA binding	S-glutathionylation of a thiol on c-Jun
	DEA/NO	0.1–1 mM	N.A.	↓ DNA binding	

^a Demonstration of cell, concentration, and stimulus specificity.

but offered no further insight. One explanation for the NO effect is that it activates JNK (95, 96). Although JNK itself may be a NO target (97), it is probably a kinase further upstream in the JNK pathway that transduces the NO signal (98).

The theme that emerges from all of these studies is one in which NO- and O₂-related molecules do not just interact with AP-1 itself. Instead, they regulate other molecular components in the signal transduction pathway. It is therefore understandable why a consensus does not exist as to whether NO is an activator or inhibitor of AP-1 or whether AP-1 mediates or counters oxidative and nitrosative stresses. That is, there is no single redox paradigm into which AP-1 can be fit. Rather, the AP-1 activation pathway seems to be capable of sensing diverse redox-related signals in a variety of signaling circuits. The nature of

these redox-related signals and the components of the circuitry remain to be elucidated.

p53, EGR-1, and other nitrosant/oxidant-sensitive transcription factors

Numerous additional mammalian transcription factors exhibit redox sensitivity (Table 4). The difficulty lies in placing the sparse data into physiological context. The tumor suppressor protein p53, for example, binds to its specific DNA sites more favorably in a reducing environment (99–103), and mutation of cysteine residues in the p53 core binding domain prevents DNA binding and p53-induced transcription (101). It also appears that Ref-1, similar to its redox role with NF-κB and AP-1, regulates p53 DNA binding and gene transactivation (104, 105). But whereas alkylation or oxida-

TABLE 4. Other redox-sensitive transcription factors^a

Transcription factor	DNA binding domain	Location of regulatory cysteine(s)
c-Myb	Helix-turn-helix	Single conserved cysteine in the DNA binding domain
p53	Loop-sheet-helix motif linked to a loop-helix motif	Redox sensitivity mapped to nine candidate cysteines in the DNA binding domain
NF-Y/CBF	Helix-loop-helix like motif	Two conserved cysteines in the DNA binding domain
SP-1	Zinc finger	Unknown
NFI/CTF	Unique motif	Single cysteine in DNA binding domain
EGR-1	Zinc finger	Unknown number of cysteines in DNA binding region
Glucocorticoid receptor	Zinc finger	Several candidate cysteines in the DNA binding, nuclear localization, and ligand binding domains
Heat shock factor	Not described	Unknown
TTF-1	Homeodomain	Two cysteines located outside the DNA binding homeodomain
USF	Helix-loop-helix	Two cysteines in the DNA binding domain
Pax-8	Paired domain	Single cysteine in the DNA binding domain
Hox B5 (Hox-2.1)	Homeodomain	Single cysteine in DNA binding domain
PEBP2	Runt domain	At least one, possibly two, cysteines in the DNA binding domain
GABPα	Helix-loop-helix	Two cysteines in the DNA binding domain and one in the dimerization domain
HLF	Basic-helix-loop-helix	Single cysteine in DNA binding domain
HIF-1α	Basic-helix-loop-helix	Single candidate cysteine in the transactivation domain

^a Location of reactive cysteines and the type of DNA binding domain.

tion of thiols inhibits DNA binding of p53 (99–103), zinc as well as thiol-reducing agents are needed to reverse the oxidative inhibition. These data suggest that at least some of the cysteines are involved in the coordination of zinc within p53 (103).

One cannot deduce from these studies a clear understanding of the molecular mechanism by which p53 is redox modulated. It has been suggested that oxidation of p53 may involve either the formation of a sulfenic acid, a mixed disulfide, or an intramolecular disulfide; however, this is only speculation (100, 102). Moreover, this explanation does not reconcile well with the increase in DNA binding seen on zinc coordination to the cysteine redox centers. An intriguing notion that zinc somehow protects the cysteines from oxidation has been forwarded (100, 101, 103), but this is not a common function for the metal and the result *in vitro* cannot easily be extrapolated to biological systems.

Zinc/cysteine interactions are also important in the redox sensitivity displayed by Sp-1, EGR-1 and the glucocorticoid receptor (GR)—members of the zinc finger family of DNA binding proteins. Both reducing conditions and zinc are required for Sp-1 and EGR-1 to bind their target DNA sites (106–108). Thus, production of ROS, which tend to oxidize thiols and eject zinc, decreases Sp-1 DNA binding and transcription of Sp-1-responsive genes in cell systems (109). This effect, however, is unlikely to be regulatory. The glucocorticoid receptor displays NO/redox-sensitive DNA binding (110, 111). The question arises as to whether these reactions with critical cysteines are regulatory or representative of nitrosative stress. In the former instance, NO-related molecules might protect thiols from oxidation through covalent modification or even promote reversible oxidative modifications that change protein structure (14). In the latter instance, however, they eject zinc and thereby disrupt the zinc finger domains that are essential for transcription factor functioning (112); it would be more difficult to restore activity in this scenario.

Notwithstanding these examples, zinc is not essential for molecular recognition of redox signals and reducing conditions do not always promote DNA binding of transcription factors. Examples of transcription factors whose binding to DNA is facilitated under reducing conditions independently of zinc include c-Myb (113), USF (114), NFI (115), NF-Y (116), HIF-1 α (117), HLF (118), PEBP2 (119), GABP α (120), TTF-1 (121, 122), and Pax-8 (122). In contrast, the DNA binding of Hox B5 is increased under oxidative conditions (123). All of these transcription factors have critical cysteine residues that alone confer the redox sensitivity. GABP α has two cysteines that regulate DNA binding and one that is required for dimer-

ization with GABP β , the trans-activating subunit (120). In Pax-8, 3 cysteines confer redox-sensitive DNA binding, which involves intramolecular disulfide formation (124). HIF-1 α has a single cysteine in the carboxyl-terminal trans-activating domain, which participates in protein–protein interactions that activate transcription, e.g., with the transcription factor, CREB binding protein (125). The GR contains reactive cysteines in the ligand binding and nuclear localization sequences distinct from those in the zinc finger (DNA binding) domain that must be reduced for transcriptional activation (126, 127). Two lines of evidence suggest that both redox and NO responsiveness are likely to be shared features of this group of transcription factors. First, NO inhibits the DNA binding of c-Myb as well as GR via interactions with thiols in structurally distinct regions of these protein (128, 129). Second, the reducing protein systems thioredoxin and Ref-1 regulate the transcriptional activity of GABP α , Pax-8, PEBP α , HIF-1 α , HLF, and the GR (117–119, 130–132), implying a general mechanism of redox control. Taken as a whole, these data make a compelling case for redox/NO responsiveness; but does this mean that the transcriptional responses under the control of these proteins are redox- or NO-regulated *in vivo*?

It is understood from the foregoing discussion that the effects of the redox state on DNA binding of transcriptional regulators *in vitro* must be placed into physiological context. That is, DNA binding assays *in vitro* may or may not relate to the cellular control mechanism *in vivo*. Take the case of the heat shock transcription factor (HSF), which is activated by reductants *in vitro* and by oxidizing conditions in cell systems (133, 134). HSF may be analogous to NF- κ B, which is activated by oxidation in the cytoplasm while reducing conditions in the nucleus promote DNA binding. In the case of p53, the *in vitro* inhibition by NO-related molecules (135) has no known correlate of physiological relevance. Indeed, induction of iNOS or treatment with nitrosothiols results in up-regulation of p53 in macrophages, fibroblasts, and epithelial cells (136, 137). p53, in turn, decreases the expression of iNOS through binding to the iNOS promoter (137). Thus, nitrosation or oxidation of p53 evidently is not occurring in these systems. It is more likely that nitrosative damage is being sustained by the DNA, which leads to p53 activation. In contrast, the inhibitory effects of NO on EGR-1 activity in cells are at least consistent with the data from *in vitro* binding studies (138, 139). These studies imply that S-nitrosylation is the mechanism of inhibition, but in fact alternative mechanisms of regulation cannot be discounted.

REDOX AND STRUCTURAL CHANGES AT THE MOLECULAR LEVEL

It is clear that most redox-active transcription factors have conserved cysteines whose modifications can sense changes in either the redox state of the cell (or subcellular compartment) or in the level of a particular redox-related effector molecule. However, our understanding of the mechanism by which modification of these cysteines can translate into molecular activation is still rudimentary. A number of innovative *in vitro* studies have attempted to elucidate these mechanisms. Even though these *in vitro* studies do not typically consider other cellular proteins that are associated with the transcription factor and which may modulate its DNA binding ability, they provide useful insights into the molecular mechanisms associated with redox signaling.

The binding of a transcription factor to DNA usually induces conformational changes in the transcription factor, the target DNA site, or both. For example, when NF- κ B p50 homodimers bind to DNA, a conformational change in the protein is observed (e.g., by circular dichroism) (140). It has been surmised that the structural change results in the formation of an α -helix in the AB loop of the p50 NH₂-terminal DNA binding domain. This AB loop is highly unstructured in the absence of bound DNA and is in the same region where the redox-active cysteines are located. Other studies have shown that NF- κ B binds to DNA only when these cysteines are reduced and that redox-related modifications of these cysteines, such as the formation of disulfides (34) or nitrosothiols (54), disrupts DNA binding ability. Furthermore, a structural variation in the DNA only occurs if the protein is bound to a specific κ B site. Similarly, the DNA-recognizing paired domain of Pax-8 is composed of helix-turn-helix subdomains, which also contain the conserved redox-active cysteines (124). The protein shows an increase in α -helical structure that is conducive to DNA binding when these cysteines are reduced. This redox-regulated activity is confined to only one of the paired domains and is controlled by the reversible formation of a disulfide bond between the conserved cysteines. These two examples do not, however, explain differences in transcriptional response to different redox-related stimuli. Rather, they suggest that no matter what redox stress the cell is exposed to, the ultimate molecular response by the transcription factor will be the same. This model, therefore, would not explain the differential binding affinities of oxidized, reduced, and S-nitrosylated NF- κ B (141), and raise the possibility of distinct structural changes in the protein that alternatively effect DNA binding.

The two-state (oxidized/reduced) model also falls short in explaining the behavior of other redox-

active transcription factors. For example, the conserved cysteines in PEBP2 have vastly different redox potentials and do not participate in disulfide bond formation. Yet both are involved in DNA binding (119). In addition, the spatial arrangement of the conserved cysteines in the GABP α does not favor the formation of disulfides (120). Rather, it has been proposed that these cysteines regulate transcription by either electrostatic interference with DNA binding, when in close proximity to the binding site or by inducing a conformational change in the protein if distant from the binding site. As a whole, such observations lead to an interesting speculation that redox-initiated differential regulation by a protein is possible. In other words, depending on the nature of the redox-related posttranslational modification (i.e., S-OH, S-SR or S-NO), different genes will be transcribed.

CONCLUSION

Many transcription factors exhibit sensitivity to NO- and O₂-related molecules. However the molecular basis of regulation has not been elucidated in any eukaryotic DNA binding protein. Some very basic questions remain. For example, it is not known whether redox sensitivity of transcriptional activators is designed to sense a change in the redox status of the cell or to recognize specific redox-related signals. It is tempting to speculate that a common site and mechanism of molecular recognition may exist whereby oxidants and nitrosants affect gene expression in the former case, while the means to discriminate among different redox-related molecules may exist in the latter case. In either scenario, critical cysteine residues in the DNA binding domains or at distant allosteric sites appear to serve in molecular recognition of the signals.

What is the molecular basis of the signal? At the simplest level, nitrosylation or oxidation of thiols regulates transcription of target genes. But in practice, each redox-related modification may have its own functional consequences. Moreover, regulation in cells may occur at multiple levels in signal transduction pathways and different incoming signals may be subject to different redox control mechanisms. Although it remains to be seen in how many ways transcription factors integrate nitrosative and oxidative signals into functional responses, it should not be forgotten that the molecular events underlying redox control of mammalian gene expression have not been elucidated in any well-defined cellular system or physiological response. **[F]**

Note added in proof: Ding and Demple (*Proc. Natl. Acad. Sci. USA*, vol. 97, 5146–5150, 2000) have recently suggested that NO may form a complex with SoxR.

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