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Forum Review

Oxidation of Zinc-Binding Cysteine Residues in Transcription Factor Proteins

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ABSTRACT

Recent results on the oxidation of cysteine residues that bind zinc in transcription factors and their analogous peptides and in related proteins and model systems are reviewed. Two classes of oxidants, the transition metals and dioxygen, hydrogen peroxide, and related species, are considered, and the role of metal ions in suppressing or enhancing Cys oxidation is a major focus. Cysteines in the zinc-bound structures of transcription factors are less susceptible to oxidation than in the metal-free form, and this appears to correlate with reduced accessibility of the thiolates to oxidants. Substitution of other metal ions for Zn(II) increases the rate of Cys oxidation, apparently through increased oxidant accessibility. Reactions that result in reversible or irreversible oxidation of these zinc-binding cysteines under biological conditions are identified in the context of deleterious implications for gene expression. Antioxid. Redox Signal. 3, 549–564.

INTRODUCTION

CONTROL OF GENE EXPRESSION is necessary for proper cell differentiation, metabolism, and response to stress, and transcription factor proteins that initiate or suppress gene expression by binding to DNA play crucial roles in regulating these cellular processes. As transcription factors use unique protein structures to bind to specific DNA sequences, reactions that alter the properties of these protein structures may be involved in controlling gene expression or initiating cytotoxicity and/or genotoxicity.

Transcription factors use a variety of structural motifs to bind to DNA, and many of these require stabilization by Zn(II) (5). The first of these Zn-binding motifs to be recognized (8, 44) were the so-called zinc fingers, which have the canonical sequence -Tyr/Phe-X-Cys-X_{2-4}-Cys-X_3-Phe-X_5-Leu-X_2-His-X_{3-5}-His- (X = nonconserved residue) that appears in up to ~30 tandem repeats in the DNA-binding domain of these proteins. Zinc coordination to the two Cys (C) and two His (H) stabilizes a $\beta\beta\alpha$ structural motif (37) (Fig. 1) that is competent to make hydrogen bonds with specific DNA bases (54). In addition to zinc fingers, other sequences that use three Cys and one His or four Cys to bind Zn(II) are known and, in some cases such as the CCHC Zn-binding motif from the nucleocapsid protein of retroviruses (17, 66), have been well studied. It is now clear that Zn(II) ions stabilize a variety of protein structures that are required for protein–DNA or protein–protein interaction (63), and that all of these involve Zn(II) binding to Cys thiolate ligands.

Zinc has certain advantages (52) over other

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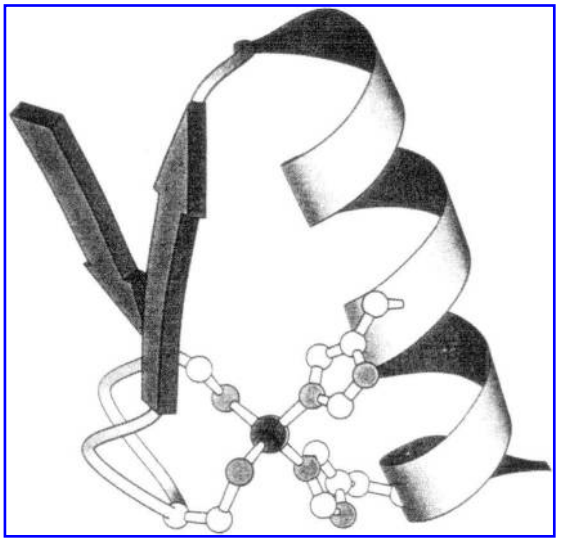


FIG. 1. Ribbon diagram of the classical zinc finger structural domain, with the two Cys and two His side chains that are coordinated to the Zn(II) ion (dark sphere) indicated by ball-and-stick representation (used with permission from 40).

metal ions as a component of proteins involved in nuclear processes, such as transcription. Foremost is its lack of redox reactivity under biological conditions. The total cellular concentration of Zn makes it fairly abundant among first row metals, although its free ion concentration in the cytoplasm is quite low, $\leq$100 p*M* (3). Zn(II) is a borderline Lewis acid, with little difference in its preference for soft (*e.g.*, Cys) or hard (e.g., Asp, Glu) protein ligands. Other properties of this metal ion include its flexibility in the type and number of ligands, although four-coordinate tetrahedral ligation is most common, and its relatively rapid ligand substitution reactions. Although it is not obvious that these latter properties are important for stabilizing protein structures, rapid ligand substitution is a requirement for the hypothesis that Zn(II) removal from transciption factors by metallothionein (MT) may be used to control gene expression (10, 85, 86). This property also allows for rapid substitution by other metal ions.

Because Zn(II) is essential for DNA recognition and binding by certain transcription factors, their Zn-binding sites may be subject to unique modifications with deleterious consequences, as originally noted by Sunderman and Barber (68). Substitution of other metal ions for Zn(II) may result in an altered protein structure that is not competent to bind DNA or has altered DNA sequence specificity. Substitution by transition metal ions may, in addition, result in redox reactions that can oxidize the transcription factor protein or possibly nearby DNA.

All known Zn(II)-binding sites in transcription factors have at least two and often four Cys ligands, which can be oxidized under physiological conditions. Usually this is a one electon/Cys oxidation and results in disulfide bonds, which can be reduced by biological reductants, such as glutathione (GSH). Disulfides are poor ligands for Zn(II), and it has been shown in several cases (2, 28, 29, 33) that oxidation of Zn-binding cysteines eliminates the ability of these transcription factors to bind to DNA, even in the presence of excess Zn(II).

The mechanism of Cys oxidation can be categorized as an outer sphere electron transfer or an inner sphere oxidation that involves direct interaction of the thiol with the oxidant. In the former case, one-electron oxidation results in a thiyl radical, which can couple with a free Cys to form the disulfide radical anion, which is a strong reductant and loses an electron to form the disulfide.

$$RSH \xrightarrow{-e^-} RS^{\cdot} + H^+ \xrightarrow{+RSH} RSSR^- + H^+ \xrightarrow{-e^-} RSSR$$

Inner sphere oxidation involves attack by the thiol nucleophile on the electrophilic oxidant. In the case of hydrogen peroxide (H_2O_2), this is believed to result in initial two-electron oxidation to the sulfenic acid, which is subsequently attacked by a free Cys to give the disulfide.

$$RSH + H_2O_2 \rightarrow RSOH + H_2O \xrightarrow{+RSH} RSSR + H_2O$$

Cysteine residues can be oxidized by different classes of oxidants under biological conditions (22, 32). One class is the transition metals, many of which undergo rapid one-electron redox reactions at physiological potentials. Others, such as chromium and molybdenum, can be involved in multiple-electron redox reactions. Another class of oxidants includes dioxy-

gen, H_2O_2, and related species, which typically are attacked by the nucleophilic thiol. However, these oxidants may be more efficient when the redox reaction is mediated by transition metal ions, such as Cu(II), Ni(II), or Co(II) (18, 35). Not only can reaction rates be accelerated, but reduction potentials can be modulated by metal coordination; for example, peroxide is an even stronger oxidant when it is bound to Cr(III) (4).

There are increasing examples of the oxidation of Cys residues in proteins to species other than the disulfide. Organic thiols can be oxidized by successive two-electron steps to the sulfenic acid (RSOH), sulfinic acid (RSO_2H), and sulfonic acid (RSO_3H) (11), and several cases where Cys residues in enzymes and proteins can be oxidized to these and related species are now known (19). As sulfinates and sulfonates cannot be reduced by biological reductants, they constitute irreversibly modified (damaged) forms of cysteine.

This article reviews the oxidation of metal-binding Cys residues in transcription factor proteins and their analogous peptides and in related proteins and model systems, including some of our recent unpublished results. The goal is to identify reactions and conditions that result in Cys oxidation at Zn-binding sites in transcription factors. Although this may be a mechanism to control gene expression (67, 79, 83), the focus here will be the deleterious implications of these reactions.

OXYGEN-BASED OXIDANTS

Dioxygen is a powerful four-electron oxidant ($\epsilon^{\circ\prime} = 0.816$ V), but a weaker two-electron oxidant ($\epsilon^{\circ\prime} = 0.28$ V) and a poor one-electron oxidant ($\epsilon^{\circ\prime} = -0.16$ V). The species resulting from the latter two electron transfer reactions, H_2O_2 and superoxide anion, are themselves a potent two-electron oxidant ($\epsilon^{\circ\prime} = 1.35$ V) and one-electron oxidant ($\epsilon^{\circ\prime} = 0.89$ V), respectively. However, these reactions are pH-dependent and, in some cases, there is a significant kinetic barrier to redox reactions involving these species, resulting in low reaction rates. For example, the ground state of dioxygen is a triplet ($^3O_2$) and that of most organic molecules (*e.g.*, thiols) is a singlet, resulting in a significant spin barrier to redox reactions. Thus, although the oxidizing abilities of superoxide, H_2O_2, and dioxygen are often invoked to explain the oxidation of biological molecules, the conditions and rates of the proposed reactions need to be consistent with the known chemical properties of the oxidant.

In most cases, living organisms have developed metal-based systems for managing the oxidizing ability of these molecules (40). This includes metalloproteins involved in O_2 transport and storage (hemoglobin, myoglobin, hemocyanin, hemerythrin), metalloenzymes that use dioxygen or H_2O_2 to oxidize biological substrates (oxidases, peroxidases), and metalloenzymes that remove superoxide and H_2O_2 by disproportionation (superoxide dismutase, catalase). Thus, metal-binding sites in proteins are likely sites for oxygen-based reactions.

Several examples are now known where metals mediate the oxidation of a protein ligand of the metal ion, and often it is a Cys thiolate ligand that becomes oxidized. In most of these cases, this is a deleterious outcome, but in at least one case this appears to be part of an essential posttranslational modification of metal-binding Cys residues. These examples have significant implications for Zn-binding sites in transcription factors, where two or more of the protein ligands are cysteine.

Metalloenzymes and metalloproteins

One example of metal-mediated oxidation of Cys ligands that has received some attention is the Ni-containing hydrogenases (H_2ase; EC 1.12.2.1) from several anaerobic bacterial species (1). The enzyme active site (Fig. 2) contains Ni(II) in a Cys-rich coordination with thiolates that bridge to an Fe(II), which has two cyanide and one carbon monoxide ligands (72). Exposure of these enzymes to aerobic conditions results in reversible or irreversible inactivation, which is associated with oxidation of the Ni-coordinating ligands. To elucidate this oxidative inactivation, several model complexes with Ni(II) in a sulfur-rich coordination have been prepared and investigated (26, 45). Exposure of these complexes to oxidants, including O_2 and H_2O_2, results in oxidation of

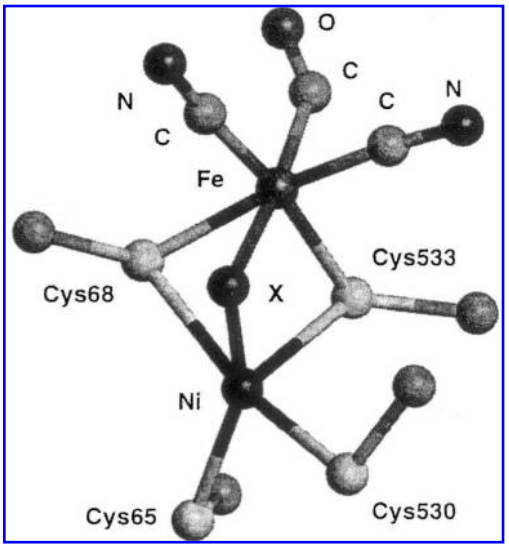


FIG. 2. Ball-and-stick representation of the NiFe active site of *Desulfovibrio gigas* H_2ase, where X = S^{2-} or O^{2-} and the other three nonprotein ligands of the Fe(II) are two cyanides and one carbon monoxide (adapted with permission from 72).

alkyl-thiolate ligands to the Ni-bound sulfoxide (sulfenate) or sulfone (sulfinate), depending on the oxidant, the thiol ligand, and the specific conditions (Fig. 3). Reactivity studies (9) and theoretical analysis (43) indicate that Ni(II) coordination enhances the nucleophilicity of the thiolate for attack on the oxidant. This reactivity, however, is not observed with analogous Zn(II) or Fe(II) complexes, suggesting an electronic contribution from the Ni(II).

Similar reactivity has been found recently for the enzyme nitrile hydratase (NHase; EC 4.2.1.84), which contains either a low-spin Fe(III) or a Co(III) and catalyzes the hydration of nitriles to the corresponding amides (81). The active site consists of the metal ion bound to three Cys residues and two peptide amides in the short sequence, -Cys^{109}-Ser-Leu-Cys^{112}-Ser-Cys^{114}-, and to an exogenous nitric oxide (NO) ligand, in an inhibited form of the enzyme (49) (Fig. 4). However, the three Cys residues are in three different oxidation states: one is a reduced thiolate (Cys^{109}), another has been oxidized to an S-coordinated sulfenate (Cys^{114}), and the third has been oxidized to an S-coordinated sulfinate (Cys^{112}). Although there is a NO ligand, it has been shown that O_2 is involved in the posttranslational oxidation of these Cys residues (51). As with H_2ase, reactivity that generates this metal coordination site has been studied with model complexes. When certain Fe(III) and Co(III) complexes with thiolate ligands are exposed to dioxygen or H_2O_2, the thiolates can be oxidized to the sulfenate and/or sulfinate, depending on the conditions (36, 71) (Fig. 5). Because of the different extent of oxidation of the three metal-binding Cys residues in NHase, metal control of the oxidation of these Cys ligands appears likely.

Another, although still poorly characterized, example of Cys oxidation involving metal ions is the Fe-containing enzyme cysteine dioxygenase (EC 1.13.11.20) that catalyzes O_2 oxidation of Cys to form cysteine sulfinic acid in the cysteine metabolic pathway (82). Although the reactive Cys in this case is an exogenous substrate, the enzymatic mechanism may involve

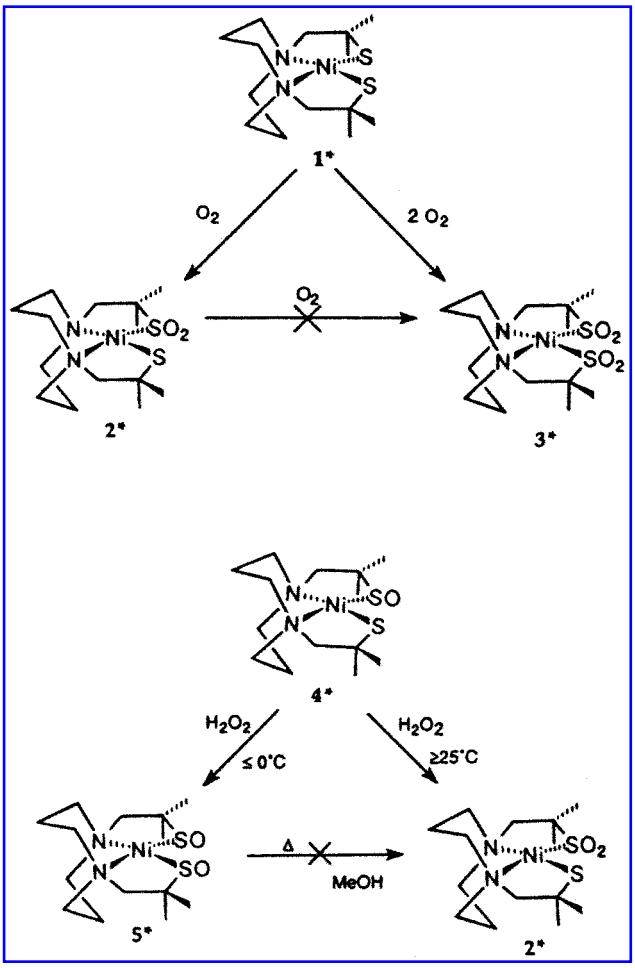


FIG. 3. Reaction schemes and structural representations of species resulting from oxidation of Ni-thiolate complexes by O_2 (top) and H_2O_2 (bottom) (adapted with permission from 9).

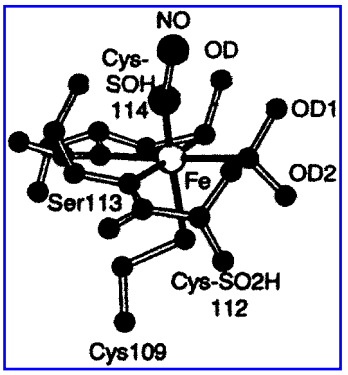


FIG. 4. Ball-and-stick representation of the Fe(III) coordination to Cys^{109} (thiolate), Cys^{112} (sulfinate), amide between Cys^{112} and Ser^{113}, amide between Ser^{113} and Cys^{114}, Cys^{114} (sulfenate) and NO in the NHase active site (adapted with permission from 49).

cysteine binding to the Fe(II), followed by reaction with dioxygen that may or may not also be bound to the iron, as has been found for other Fe-containing dioxygenases (58).

Although the above examples involve Ni(II), Fe(II), Fe(III), and Co(III), there are cases where Zn(II) coordination to Cys residues or other biological thiolates modulates their reactivity. The *Escherichia coli* Ada protein, which removes methyl lesions from DNA, uses a Zn-bound cysteine thiolate as a nucleophile to remove methyl groups from the phosphate backbone of DNA (47). A growing class of enzymes, including the cobalamin-dependent and -independent methionine synthases, contains a Zn(II) that binds thiol species (*e.g.*, homocysteine, coenzyme M) involved in alkyl transfer reactions, and the Zn(II) appears to play an essential role in thiol reactivity (55). Although a deprotonated thiolate is a better nucleophile than the protonated thiol, which is the predominant protonation state of Cys near neutral pH, the relative nucleophilicity of a Zn-bound Cys thiolate at neutral pH is currently not well known.

Perhaps most relevant to the oxidation of Zn-coordinated Cys residues in transcription factors is oxidation of the cysteines of metallothionein (MT) (42). This small Cys-rich protein binds seven dipositive metal ions [Zn(II), Cd(II)] in two ~30-residue domains using exclusively Cys thiolate ligands. Exposure of Cd_7-MT or Zn_7-MT to aerobic conditions results in disulfide-linked dimer species that can be converted back to the monomer by treatment with dithiothreitol (DTT), but these species begin to appear only after several days, indicating that the metal-bound Cys are protected from oxidation by O_2 (53). The reactions of Zn_7-MT with different oxygen species, including $OH^{\cdot}$, O_2^-, and H_2O_2, have been studied. Whereas the protein reacts near the diffusion-controlled limit with $OH^{\cdot}$ ($\sim 10^{12}\ M^{-1}\ s^{-1}$ at 298 K), it reacts much slower with O_2^- ($\sim 10^5\ M^{-1}\ s^{-1}$ at 298 K), even slower than the reaction of GSH with O_2^- (70). Exposure of Zn_7-MT or Cd_7-MT to ionizing radiation, which generates these two species, results in Cys oxidation and loss of bound Zn(II) or Cd(II), but metal binding can be restored by treatment with DTT, indicating oxidation only to the disulfide. Finally, it has been shown that H_2O_2 oxidizes the Cys residues of Zn_7-MT and liberates Zn(II), but at a rate that is slow relative to that of O_2^- (20). Treatment of cells with H_2O_2 results in Zn(II)

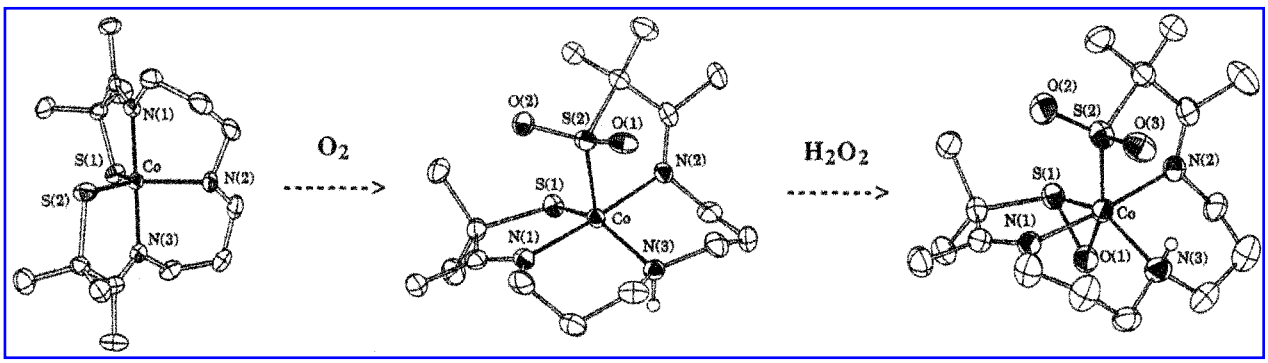


FIG. 5. X-ray diffraction structures (Oak Ridge thermal ellipsoid program plots) of a Co(III)-bis(thiolate) complex, the Co(III)-sulfinate/thiolate complex that results from exposure to air, and the Co(III)-sulfinate/η^2-sulfenate complex that results from subsequent treatment with H_2O_2 (adapted with permission from 36).

mobilization from MT and oxidation of the MT Cys residues, possibly to species beyond the disulfide oxidation level (59).

Transcription factor proteins

Although the role of Zn(II) ions bound to transcription factors appears to be exclusively structural, the above examples suggest that the chemical properties of these Zn(II) coordination sites may have biological or toxicological significance. First, as in the example of the Ada protein and related enzymes, enhanced nucleophility of a Cys thiolate ligand, relative to the protonated thiol, may be a consideration. Second, if a transition metal ion replaces the Zn(II), then enhanced reactivity of Cys ligands with oxidants through a metal-mediated mechanism may be possible, as was found for H_2ase and NHase. In fact, other metal ions [Co(II), Cd(II), etc.] often have been substituted for Zn(II) in studies of zinc fingers and other Zn-binding domains (21, 24), and a number of studies have characterized the DNA-binding properties of metal-substituted transcription factor domains (48, 57, 69, 73). Less well studied, however, is the reactivity of these sites when transition metal ions are bound in place of Zn.

Results from gel mobility shift assays (GMSA) have shown that oxidizing agents (H_2O_2, diamide, etc.) can eliminate DNA binding by transcription factors that contain zinc fingers (2, 28, 29, 33). This effect can be reversed by thiol-reducing agents and, in at least one case, it was reported that Zn protects the Cys thiols from oxidation (33). We have investigated these observations with studies of a classical zinc finger peptide, the third zinc finger of the transcription factor Sp1 (Sp1-3) (56). NMR studies have shown that this peptide folds into the canonical zinc finger structure (50), supporting its use as a model for the chemical and structural properties of a zinc finger sequence in a protein. Initially we found that metal ions modulate the rate of Cys oxidation under aerobic conditions, with loss of free thiols increasing in the order Zn(II) $<$ apo (metal-free form) $\approx$ Co(II) $<$ Ni(II). Monitoring the thiolate-to-Ni(II) charge transfer transitions of Ni-Sp1-3 showed that Ni(II) coordination to the Cys thiols was completely eliminated within 2 h after exposure to a threefold excess of H_2O_2.

The species resulting from oxidation of Sp1-3 were then investigated (80). Initially, HPLC was used to analyze the peptide products after exposure to H_2O_2 or O_2. These results showed, as found previously by free thiol assays, that Zn(II) protected the Cys thiols from oxidation to the disulfide form, but the Ni-Sp1-3 peptide was substantially oxidized to the disulfide within $\frac{1}{2}$ h of exposure to a threefold excess of H_2O_2 (Fig. 6). Further, a second oxidized species appeared within this period and it became a significant component within a few hours. Analysis of this peptide showed that it had a new IR vibration at 1,077 cm^{-1} and a mass consistent with addition of an oxygen atom to the disulfide oxidized form, results that are consistent with formation of a thiolsulfinate (30, 62) in this oxidized zinc finger. This species could not be reduced by treatment with DTT. Although unprecedented in proteins or peptides, a thiolsulfinate is found in β-lipoic acid (61), the lacrimator allicin from onion and garlic (6), and the antitumor antibiotic natural product leinamycin (23).

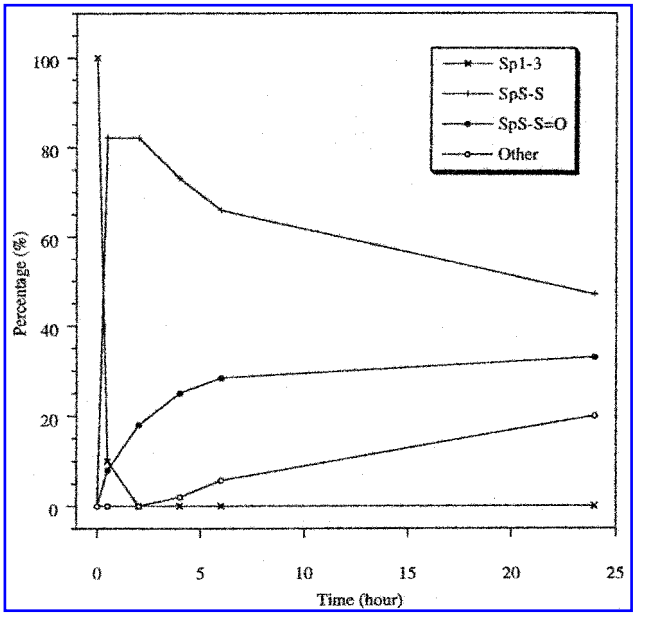


FIG. 6. Plot of the relative percentages of peptide species at 0.5, 2, 4, 6, and 24 h after treatment of Ni-Sp1-3 with threefold excess H_2O_2 (Sp1-3 = reduced peptide, SpS-S = disulfide oxidized peptide, SpS-S=O = thiolsulfinate oxidized peptide).

Peptide analysis by HPLC was used to quantify the oxidized Sp1-3 species under various conditions. As shown in Table 1, both the amount and distribution of oxidized products are modulated by the metal ion, with Zn(II) and Cd(II) providing protection, but other metal ions enhancing oxidation to the thiolsulfinate. This latter group includes Co(II), which binds to the peptide in a tetrahedral coordination (56), analogous to that of Zn(II) and Cd(II) (50).

A more detailed investigation with Ni(II) showed that it is capable of affecting thiolsulfinate formation in Sp1-3 with O_2 directly (80). A number of control experiments examined this reactivity and formed the basis of a mechanism (Fig. 7) for this four-electron oxidation of the two Cys residues of this classical zinc finger. Initial two-electron oxidation results in the disulfide and a Ni(II)-bound peroxo species, as has been implicated in studies of DNA oxidation (39, 41, 46) and protein cross-linking (7, 25) and cleavage (16) by Ni(II)-peptides and oxidants. Subsequent attack by the Ni-stabilized peroxo leads to further two-electron oxidation to the thiolsulfinate.

Recent kinetic modeling of the H_2O_2 reaction with Ni-Sp1-3 indicates that there may be a minor direct pathway from the reduced peptide to the thiolsulfinate species (B.D. Feldman and D.E. Wilcox, unpublished results). This and other observations suggest that there may be an alternate, and yet untested, interpretation of these data. This peptide has a single Met residue, and the putative thiolsulfinate species could be a peptide where this residue has been oxidized to the methionine sulfoxide, which would have similar mass spectral and vibrational signatures. The degree of Met oxidation with different metals could depend on differences in solvent accessibility of this residue in the metal–peptide complex. However, the inability of DTT to reduce this peptide and the several precedents for oxygen atom addition to alkyl thiols coordinated to transition metal ions provide support for thiolsulfinate formation.

A recent study by Baldwin and co-workers (75) used mass spectrometry and GMSA to investigate H_2O_2 oxidation of the DNA-binding domain of the estrogen receptor (ER), a member of the homone receptor family of transcription factors. The DNA-binding domain of ER, like that of the glucocorticoid receptor (GR) and other homone receptors, contains two Zn-binding motifs, one that interacts with DNA and another that is involved in protein dimerization (Fig. 8). This study showed that the latter Zn-binding site was more susceptible to Cys oxidation, loss of Zn(II), and elimination of protein dimerization, which would destabilize the complex with its DNA recognition sequence, the ER element (ERE) (Fig. 9). Although the oxidized cysteines can be reduced by DTT under

TABLE 1. QUANTITATIVE REVERSE-PHASE HPLC ANALYSIS OF PEPTIDE PRODUCTS* FROM 24-H REACTIONS OF Sp1-3 WITH H_2O_2 AND METAL IONS

	Peptide percentage			
Reaction†	*Sp1-3*	*SpS-S*	*SpS-S=O*	*Other*‡
Sp1-3, H_2O_2	—	80	20	—
Sp1-3, H_2O_2, Zn(II)	56	36	8	—
Sp1-3, H_2O_2, Ni(II)	—	45	30	25
Sp1-3, H_2O_2, Cd(II)	22	60	18	—
Sp1-3, H_2O_2, Co(II)	—	70	30	—
Sp1-3, H_2O_2, Cu(II)	—	67	33	—
Sp1-3, H_2O_2, Fe(II)	—	67	33	—
Sp1-3, H_2O_2, Mn(II)	—	68	21	11

*Sp1-3, SpS-S, and SpS-S=O are the reduced, disulfide, and thiolsulfinate forms of the Sp1-3 peptide, respectively. The mobile phase contains 0.1% trifluoroacetic acid, and metal ions do not remain bound to the peptides during chromatographic separation.

†Each reaction consisted of ~60 μM Sp1-3, ~200 μM H_2O_2, and, where indicated, 66 μM metal ion.

‡Sum of minor species detected by HPLC.

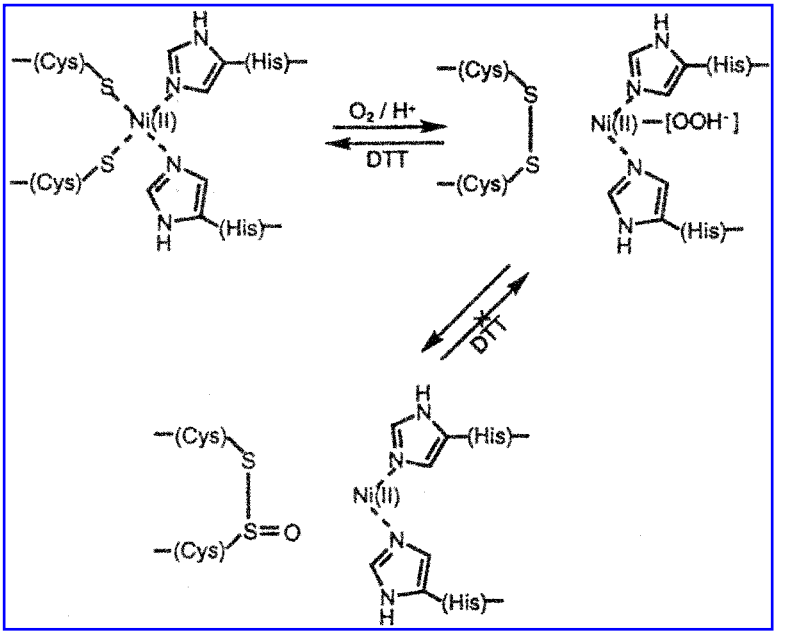


FIG. 7. Mechanism for the formation of thiolsulfinate upon treatment of Ni-Sp1–3 with dioxygen (used with permission from 80).

in vitro conditions, it is suggested that they may remain oxidized under certain *in vivo* conditions (*e.g.*, hypoxia/reperfusion of growing tumors), leading to loss of ER-controlled gene expression. This domain has several Met residues, and mass spectral evidence for oxygen atom addition was interpreted as Met oxidation to methionine sulfoxide, although the possibility of thiolsulfinates does not appear to have been considered.

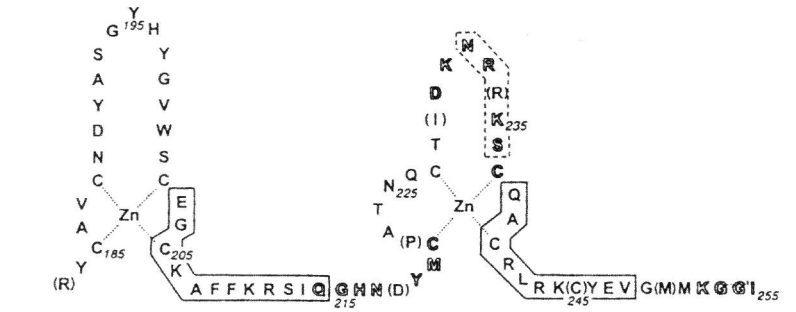
Hydroxyl radicals capable of oxidizing protein residues and DNA can be generated by the Haber-Weiss reaction cycle involving Fe(II), H_2O_2, and a reductant (27). This reactivity has been reported for the iron-substituted DNA-binding domain of ER (15). Incubation of the Fe(II)-substituted protein, ERE, H_2O_2, and ascorbate resulted in DNA cleavage patterns consistent with hydroxyl radical formation. Extensive oxidation of the Fe(II)-binding cysteines and other residues of the protein would be expected under these conditions, but apparently was not investigated.

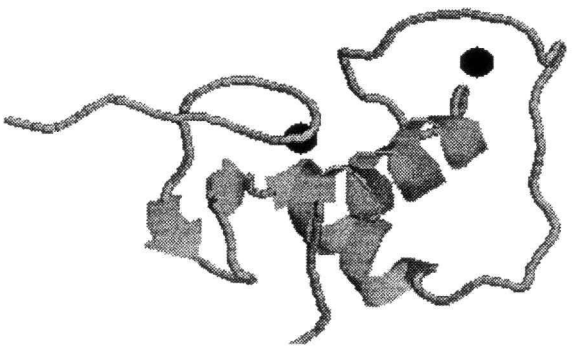
METAL-BASED OXIDANTS

One hallmark of transition metals is their redox reactivity, and many metal ions [*e.g.*, Cu(II), Fe(III), Co(III), Mn(III)] are capable of oxidizing cysteine ($\epsilon^{\circ\prime} = -0.22$ V). Thus, direct metal oxidation of Cys residues in Zn-binding sites of transcription factors needs to be considered.

Relevant to this review, it has been shown that a 10-fold excess of Cu(II) oxidizes the Zn-binding Cys residues of the HIV retrovirus nucleocapsid p7 protein, resulting in three specific disulfide bonds among the six cysteines (84). The concentrations of essential transition metals, such as Cu, Fe, and Mn, however, are tightly regulated, and they are typically bound to metal-dependent enzymes or to carrier (chaperone) proteins. For example, it has been shown recently that there is essentially no free cytoplasmic Cu in yeast cells (60). Further, the reducing intracellular environment ensures that any free metal ions are in a reduced oxidation state [*e.g.*, Fe(II), Mn(II)] and are unable to serve as an oxidant.

For certain toxic metal ions, redox reactions may be key to their deleterious effects. A well studied example is the toxic and carcinogenic Cr(VI), which exists as chromate, CrO_4^{2-}, and is imported into cells by the sulfate/phosphate anion transport pathway. As Cr(III) is a stable oxidation state, Cr(VI) can participate in redox reactions involving up to three electrons. This





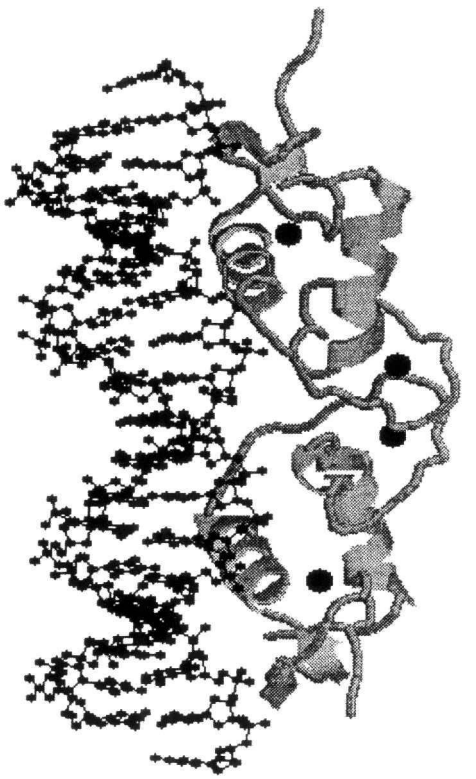


FIG. 8. Structural properties of the DNA-binding domain of ER: primary sequence, with residues in α helices indicated by boxes (**top**); ribbon diagram of the solution structure, as determined by NMR spectroscopy (**middle**) (adapted with permission from 64); ribbon diagram of the dimer complex with the ERE double-stranded DNA oligonucleotide, as determined by x-ray crystallography (**bottom**) (adapted with permission from 65). Zn(II) ions are indicated by black spheres.

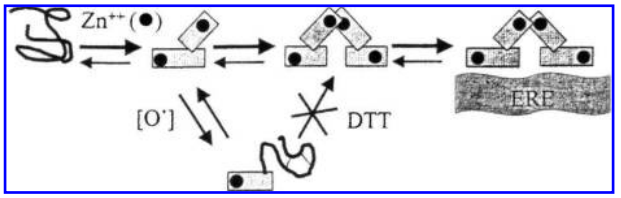


FIG. 9. Representation of the effects of Cys oxidation at the second Zn-binding site of the DNA-binding domain of ER (used with permission from 75).

redox reactivity of chromium has been associated with damage to DNA, either directly or indirectly by radicals generated from chromate redox reactions with other species in the cell (31).

Chromate interaction with cellular constituents has been reviewed by Connett and Wetterhahn (12), who subsequently investigated Cr(VI) reactions with biologically relevant reductants (13, 14), including thiols. Their results demonstrated that chromate is reduced preferentially by those reductants that react more rapidly (kinetic control). In the reaction of chromate with GSH, the most abundant cellular thiol, they showed that a Cr(VI)-glutathione thioester forms rapidly, but is reduced more slowly and may have sufficient lifetime to interact with other cellular species.

We and others have investigated the interaction of Cr(VI) with the small Cys-rich protein MT, particularly its native cellular form, Zn_7-MT. In a tissue culture model, we found that cells with elevated levels of MT are more sensitive to chromate toxicity, and this correlates with increased levels of cellular oxidants that may arise from chromate reactions with MT (76). However, our studies of the direct reaction between chromate and MT showed that only apo MT, with its 20 free thiols, reduced chromate at an appreciable rate, and that some Cr remained bound to the protein (T.S. Tylee and D.E. Wilcox, unpublished results). Others have reported that Cr(VI) is reduced by Zn_7-MT, but at a relatively slow rate (34), and that Cr remains bound to the protein. Thus, in the case of MT, Zn(II) coordination protects the Cys thiols from redox reactions with chromate.

Recently, we have investigated the interaction of chromate with a peptide (GR-2) corresponding to the second Zn-binding site in GR (A.D. Schenk and D.E. Wilcox, unpublished results). Kinetics of the reduction of chromate by apo GR-2 were analyzed in parallel with kinetics of the reduction of chromate by GSH, and significant differences were found. In contrast to the small thiol, which has a biphasic kinetic profile that is consistent with the following mechanism (13),

$$\mathrm{GSH + Cr(VI) \rightleftarrows GS\text{-}Cr(VI) + H^+}$$

$$\mathrm{GS\text{-}Cr(VI) + GSH \rightarrow GSSG + Cr(IV) + H^+}$$

chromate reduction by GR-2 is first order in Cr(VI) and significantly faster. This is consistent with a rapid intramolecular three-electron redox reaction that generates Cr(III), which is inert to ligand substitution and may remain bound to the peptide. Although, the intermediacy of Cr(V) and/or Cr(IV) oxidation states is not yet known and the peptide products have not been determined, a bound chromium would be expected to disrupt a Zn-binding site in transcription factor proteins.

Interaction of chromium with a classical zinc finger site has been modeled recently in a study of the reactions of the Zn(II)-thiolate complex, bis(*O*-ethyl-L-cysteinato-*N*,*S*)Zn(II), with chromate, a Cr(V) complex, and a Cr(IV) complex (38). The exclusive product in the case of chromate and a major product with the other Cr species is the disulfide oxidized ligand (Fig. 10). The reaction rates, however, are approximately two orders of magnitude slower than analogous reactions with the free ligand, further supporting a role for Zn(II) in protecting the thiolates from oxidation.

SUMMARY AND PERSPECTIVE

Several in vitro studies have demonstrated that oxidation of Zn-binding Cys residues in transcription factor proteins results in loss of metal binding ability and loss of function. It is increasingly clear, though, that these Cys residues are more resistant to oxidation when they are coordinated to Zn(II). This contrasts, however, with enzymes such as Ada protein and methioneine synthases where Cys residues and other thiol ligands of Zn(II) are essential

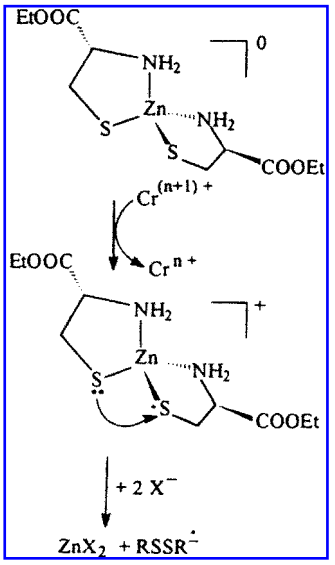


FIG. 10. Structural representation of the initial one-electron redox reaction between bis(*O*-ethyl-L-cysteinato-*N*,*S*)Zn(II) and Cr(VI), Cr(V), or Cr(IV) (used with permission from 38).

nucleophiles. This difference in reactivity likely originates from both structural and electronic features of the protein coordination site, as has been found in model studies. For example, nickel-thiolate complexes analogous to those in Fig. 3, but lacking the two methyl groups adjacent to each sulfur, react more rapidly with O_2, and this is attributed to reduced steric constraints (9), as well as altered Ni(II) electronic properties (26).

In metalloenzymes, the protein provides a unique tertiary scaffolding and environment for the catalytic metal site, and those enzymes that use a Zn-stabilized thiolate nucleophile require accessibility by substrates. In transcription factors, however, Zn(II) ions stabilize independently folded protein structures that are competent to interact with other macromolecules, and Cys accessibility is not required at these sites. This is supported by qualitative differences in Cys accessibility, as shown in Fig. 11. The Zn-coordinated Cys sulfurs of the Ada protein are noticeably more visible than are those of MT, Sp1-3, and the DNA-binding domain of ER. Consistent with the results of Baldwin and co-workers (75), the Cys sulfur ligands of the second Zn(II) (protein dimerization site) in the ER DNA-binding domain are more visible (solvent accessible), and thus more easily oxidized, than are those of the first Zn(II) (DNA binding site).

Electronic factors can also influence the susceptibility of Zn-bound Cys residues to oxidation. These can affect both the Cys reduction potential, thereby altering outer sphere electron transfer, and the Cys nucleophilicity, thus modulating its interaction with electrophilic oxidants. Recent model studies have addressed the nucleophilicity of Zn-bound cysteines in the Ada protein and related enzymes, and examined how electron donation by other ligands affects thiolate reactivity. Wilker and Lippard showed that the relative rate of nucleophilic attack by phenylthiolate, $^{-}$SPh, on $(MeO)_3PO$ to form the thioether, MeSPh, was $^{-}SPh > [Zn(SPh)_4]^{2-} > [Zn(SPh)_3(MeIm)]^{1-} > Zn(SPh)_2(MeIm)_2$, (MeIm = methylimidazole) (77), but this reactivity was found to correlate with the amount of free thiolate in equilibrium with the Zn-bound thiolate (78). A related model study recently provided evidence for nucleophilic reactivity of Zn-coordinated thiolates that was also modulated by properties of the other donor ligands (74). Both studies, and recent results with site-specific mutants of a cobalamin-independent methionine synthase (87), support the prediction that Zn(II) sites with more electron-donating Cys ligands have thiolates that are better nucleophiles and are expected to be more reactive with electrophilic oxidants. Thus, assuming similar oxidant accessibility, Zn-bound cysteines in ER, GR, and other members of the steroid receptor family should be more susceptible to oxidation than the cysteines in classical zinc finger sites.

Substitution of another metal ion for Zn(II) will perturb both structural and electronic properties of Zn-binding sites in transcription factors. First, this results in an altered protein structure with cysteines that are likely to be more exposed and susceptible to oxidation. This structural perturbation could be manifested in the equilibrium protein structure or in the protein dynamics at the Zn-binding site. Second, a different metal ion will perturb the electronic factors, affecting cysteine reactivity and possibly introducing new metal-based reactivity. For a similar ligand set, Wilker and Lippard found the relative rate of thiol nucleophilic attack on $(MeO)_3PO$ to be [Zn

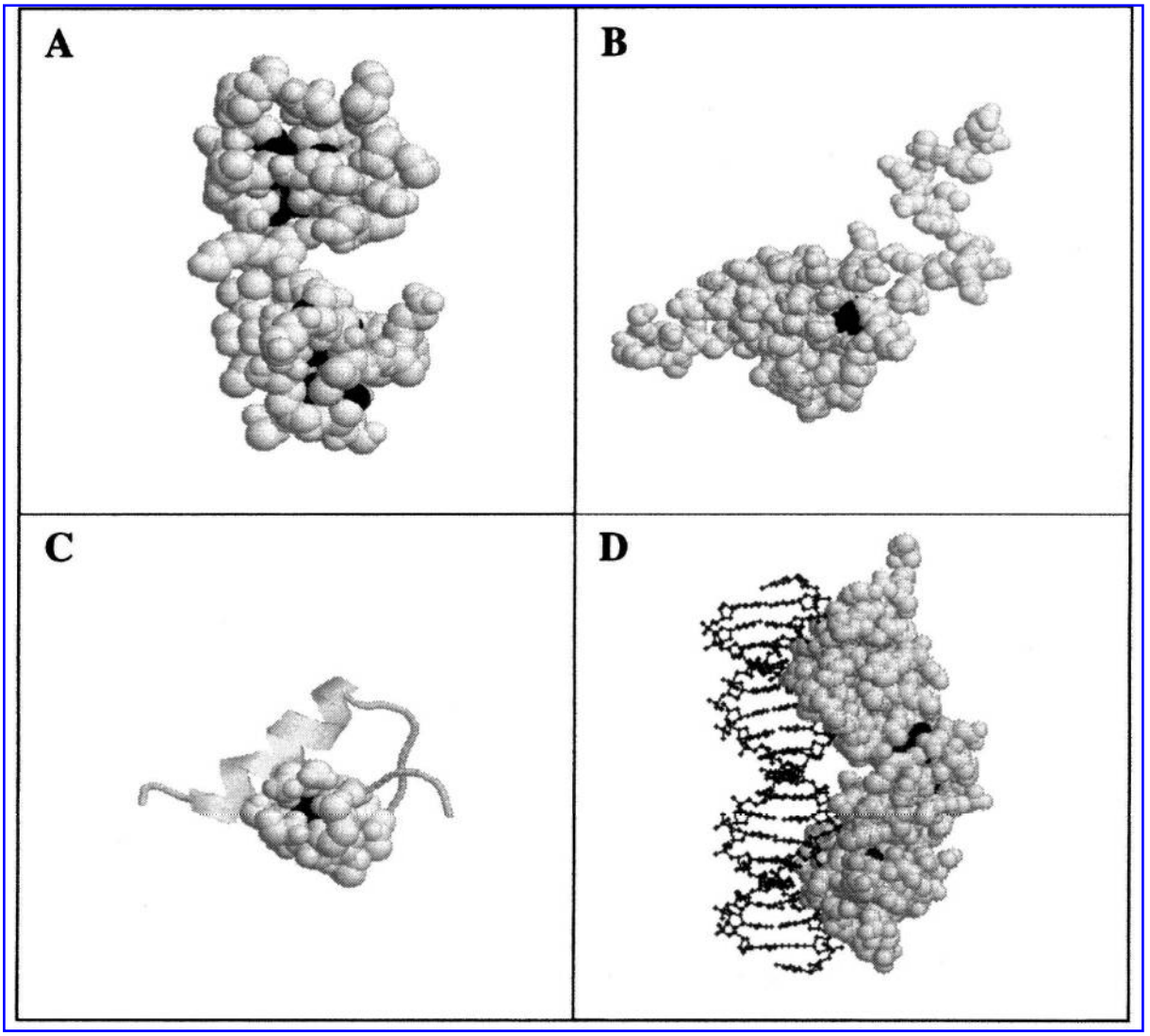


FIG. 11. Space-filling models. (**A**) Rat Cd_5,Zn_2-MT-2 (PDB file 4MT2); (**B**) *Escherichia coli* Ada protein (PDB file 1ADN); (C) third zinc finger of human Sp1 with space filling only for atoms within 5 Å of the Zn(II) (PDB file 1SP1); (**D**) human ER DNA-binding domain bound as a dimer to its duplex DNA recognition sequence (PDB file 1HCQ). In each structure, the Cys sulfur atoms bound to Zn(II) [or Cd(II)] are dark and other atoms are light.

$(SPh)_4]^{2-} > [Co(SPh)_4]^{2-} \approx [Cd(SPh)_4]^{2-} >>$ HSPh (equivalent to apo), where reactivity correlated with the amount of free thiolate anion (78). However, we found the relative rate of Cys oxidation of the Sp1-3 zinc finger peptide to be apo $\approx$ Co(II) > Cd(II) > Zn(II) (56). As metal–thiolate bonding has a similar influence on dissociation of the thiolate and thiolate nucleophilicity when it is bound to the metal, it appears that electronic effects do not predominantly influence the oxidation of zinc finger cysteines. Thus, oxidant accessibility appears to be a key factor in the oxidation of Zn-bound Cys residues of transcription factor proteins.

The product of Cys oxidation in these proteins is generally a disulfide that is a poor ligand for Zn(II) and other metal ions, but can be reduced under cellular conditions. However, more highly oxidized Cys species, such as sulfinate, sulfonate, and oxidized disulfides, can result, and we have provided evidence for thiolsulfinate formation in a classical zinc finger peptide. These species should have a higher toxicological impact because, at least in the case of sulfinate and sulfonate (cysteic acid), they

cannot be reduced to the Zn-binding free Cys under physiological conditions. Although the NHase active site and model complexes show that sulfenates and sulfinates remain bound to transition metal ions through the sulfur, it is unlikely that transcription factor proteins would remain competent if these oxidized species are formed at essential Zn-binding sites.

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ABBREVIATIONS

apo, metal free; DTT, dithiothreitol; ER, estrogen receptor; ERE, estrogen receptor element; GMSA, gel mobility shift assay; GR, glucocorticoid receptor; GSH, glutathione; H_2ase, hydrogenase; H_2O_2, hydrogen peroxide; MT, metallothionein; NHase, nitrile hydratase; NO, nitric oxide.

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