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Comprehensive Invited Review

Zinc Coordination Environments in Proteins as Redox Sensors and Signal Transducers

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ABSTRACT

Zinc/cysteine coordination environments in proteins are redox-active. Oxidation of the sulfur ligands mobilizes zinc, while reduction of the oxidized ligands enhances zinc binding, providing redox control over the availability of zinc ions. Some zinc proteins are redox sensors, in which zinc release is coupled to conformational changes that control varied functions such as enzymatic activity, binding interactions, and molecular chaperone activity. Whereas the released zinc ion in *redox sensors* has no known function, the redox signal is

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transduced to specific and sensitive zinc signals in *redox transducers*. Released zinc can bind to sites on other proteins and modulate signal transduction, generation of metabolic energy, mitochondrial function, and gene expression. The paradigm of such redox transducers is the zinc protein metallothionein, which, together with its apoprotein, thionein, functions at a central node in cellular signaling by redistributing cellular zinc, presiding over the availability of zinc, and interconverting redox and zinc signals. In this regard, the transduction of nitric oxide (NO) signals into zinc signals by metallothionein has received particular attention. It appears that redox-inert zinc has been chosen to control some aspects of cellular thiol/disulfide redox metabolism. Tight control of zinc is essential for redox homeostasis because both increases and decreases of cellular zinc elicit oxidative stress. Depending on its availability, zinc can be cytoprotective as a pro-antioxidant or cytotoxic as a pro-oxidant. Any condition with acute or chronic oxidative stress is expected to perturb zinc homeostasis. *Antioxid. Redox Signal.* 8, 1419–1441.

I. INTRODUCTION

In protein evolution, the functional potential of cysteine (Cys) as a nucleophile, redox-active amino acid, and metal ligand (68) is exploited with particular dynamics. Cysteine occurs more frequently in proteins from organisms with higher complexity (151). It has not reached its equilibrium frequency, which is lower than expected and still increasing (26, 102). These observations suggest an on-going process of utilizing the functional potential of cysteine. Within protein sequences, the spacing of cysteines is nonrandom. Cysteine has a high tendency to occur in Cys–X–X–Cys motifs that are involved in redox reactions and metal binding (151). In the interactions of cysteine with zinc, which are the focus of this review, each partner endows the other with particular characteristics that are the basis for a variety of newly discovered cysteine-dependent redox functions of zinc proteins (139).

II. REDOX-INERT ZINC AND REDOX SIGNALING

A. Zinc/cysteine coordination environments in proteins

Zinc coordination to the sulfur of cysteine occurs in catalytic sites of enzymes and in structural sites (8, 209, 210). When a cysteine ligand is present in catalytic zinc sites, the donor atoms usually are the sulfur from one or two cysteines with additional oxygen (Glu,Asp) and/or nitrogen (His) ligands, or the sulfurs from three cysteines, and a water molecule. In sites with four ligands, zinc can have one, two, three, or four cysteine ligands. Generally, histidine ligands complete a tetrahedral coordination (Fig. 1). These sites comprise structural zinc sites and the catalytic zinc sites in the zymogen form of matrix metalloproteinases where a cysteine binds as a fourth protein ligand. In some structural sites with three cysteines, the fourth ligand is a side chain from glutamate or aspartate (9). Sequence data mining established that about 3% of the estimated 32,000 human genes encode for proteins with structural zinc sites and cysteine ligation, generically referred to as zinc finger proteins. The metaphor that "zinc galvanized biology" was introduced to describe the great number of zinc proteins with cysteine coordination and to emphasize the significance of zinc in these proteins (16). Zinc fingers organize protein domains used in DNA/RNA, protein, or lipid recognition (117). In many zinc finger proteins, two zinc ions organize the structure of protein domains. In some of these double zinc motifs, the ligands do not bind to the zinc ions in the order in which they occur in the sequence. Rather, they bind the first zinc, then the second, and finally complete either the coordination of the second zinc and then that of the first (interleafed), or that of the first and then that of the second (140) (intertwined) (Fig. 2). A triple zinc motif organizes the TAZ2 domain in the transcriptional adaptor protein CBP (49). In clustered arrangements, zinc is also surrounded by four ligands, but because cysteine sulfur serves as a bridging ligand, the total number of ligands is less than four times the number of zinc ions. Zinc/thiolate clusters with one, two, three, and five ligand bridges are known (Fig. 3). Individual zinc ions in these clusters can be bound by one, two, or three bridging sulfurs. In this way, and based on the deceivingly simple tetrahedral coordination motif, zinc coordination establishes remarkable control over protein tertiary, quaternary, and quinary[1]

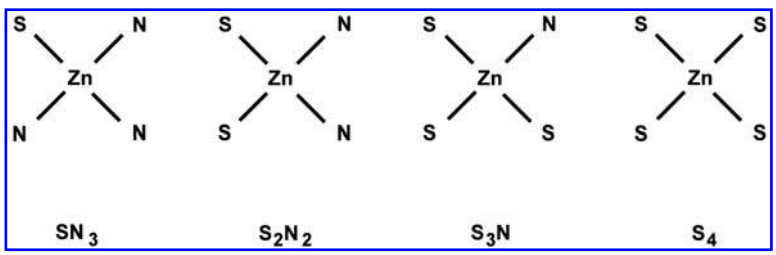

FIG. 1. The four tetracoordinate zinc motifs with cysteine ligands and a single zinc site per protein domain. *From left to right*: catalytic zinc in the zymogen form of matrix metalloproteinases, classic zinc finger, and motifs that in some proteins have been referred to as zinc box, and zinc ribbon. *S* and *N* indicate sulfur and nitrogen coordination by cysteine and histidine, respectively.

[1]The term quinary structure is used here for heterologous protein–protein interactions (191). The term was originally introduced to describe the organization of sickle cell hemoglobin into fibers and tubulin units in microtubules (53).

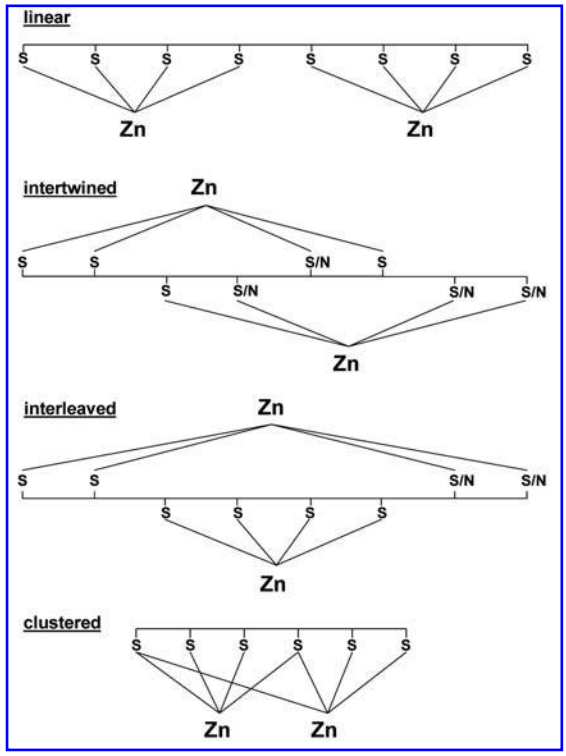

FIG. 2. Arrangement of double zinc motifs in protein domains with cysteine ligands. Linear arrangement such as in nuclear receptors or in the LIM domain, intertwined such as in RING, FYVE, and PHD domains, interleaved such as in DnaJ and the GCM domain, and clustered such as in Gal4 type of fungal transcription factors. In terms of the order in which cysteine pairs bind the first (I) and second (II) zinc ion, the linear arrangement is represented by 1_I-2_I-3_{II}-4_{II}, intertwined by 1_I-3_I-2_{II}-4_{II}, and interleaved by 1_I-4_I-2_{II}-3_{II}. S/N indicates either cysteine or histidine coordination.

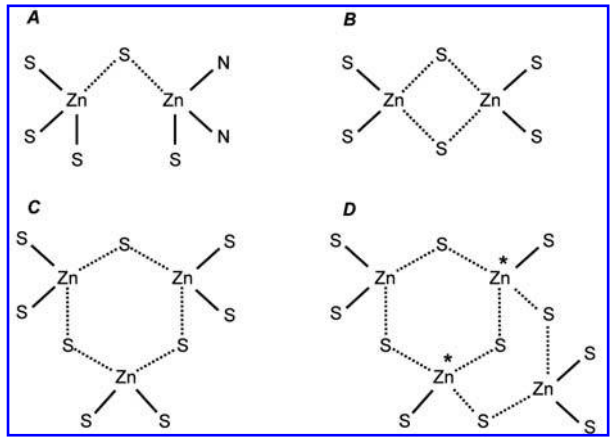

FIG. 3. Coordination of zinc in zinc/thiolate clusters. All zinc ions are tetracoordinate but the number of ligands is less than four times the number of metals due to the use of bridging sulfur ligands (*dashed lines*) in addition to terminal sulfur ligands. Examples: (**A**) V(D)J recombination-activating protein Rag1 (13); (**B**) Gal4-type fungal transcription factors (166); (**C**) N-terminal domain of MT, but also pre-SET domain of histone lysine methyltransferases (227); (**D**) C-terminal domain of MT, in which two zinc ions are bound by one terminal ligand only (*).

structure (138,140,141). In this review, it will become evident that many zinc/sulfur (Zn/S) coordination sites are dynamic structures that have other than solely structural functions.

B. Zinc proteins as redox proteins

In biology, zinc ions always remain in the Zn^{2+} state and are **not** redox-active. Consequently, zinc proteins have been considered to be redox-inert. However, because the amino acid cysteine is redox-active, zinc coordination environments with cysteine ligands have the remarkable property that the sulfur ligands can be oxidized and then reduced again with concomitant release and binding of zinc (143) (Fig. 4). Thus, the cysteine ligand confers redox properties on the complex, linking some zinc proteins to redox signaling (143). In contrast to thiol/disulfide equilibria and redox reactions at single critical cysteines in proteins, redox reactions at Zn/S centers release zinc, and the released zinc can have additional functions. This mechanism enlarges the repertoire of cysteine-based regulatory switches (163) and is referred to as a redox zinc switch (Fig. 4) (139). It enables a link between redox and zinc metabolism. Tipping the redox balance to oxidative or reductive conditions increases or decreases the availability of zinc (Fig. 5). When zinc binds to four cysteines, zinc forms a different brace than two disulfide bonds because four attachment points at the zinc ion generate less freedom of motion of the polypeptide than the joining of four cysteines through two disulfides. Unlike the oxidation of a single cysteine residue, but similar to breaking disulfide bonds, zinc release from Zn/S sites can have large effects on protein conformation (71). Disulfide bond formation requires an oxidative environment. Therefore, disulfide bonds are frequent in extracellular proteins, but rare in proteins that

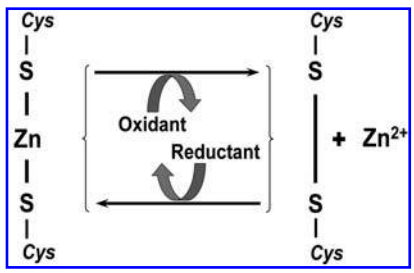

FIG. 4. Redox zinc switch. The cysteine/cystine interconversion is coupled to another redox pair and accompanied by zinc release and binding. Oxidation of the sulfur (cysteine) ligand of zinc sites mobilizes zinc, while reduction of the oxidized sulfur (cystine) induces zinc binding. This molecular mechanism links redox metabolism and zinc distribution. When the sulfur is oxidized to an oxidation state higher than the one in sulfinic acid, the reaction is irreversible.

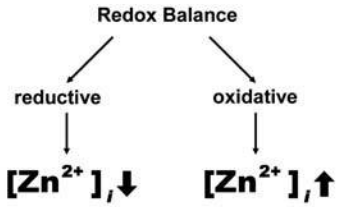

FIG. 5. Changing the redox balance changes the availability of cellular zinc. An oxidative environment increases the availability of zinc (cellular available "free" zinc, $[Zn^{2+}]_i$), while a reducing environment decreases its availability.

reside in the reducing environment of the cytosol (23). Thus, zinc can form a brace in the reducing environment of the cell, a structural feature that may account for its widespread use in proteins. Full reversibility of redox reactions at Zn/S sites with concomitant release and binding requires that zinc is available for binding. However, zinc is not freely available in the cell. Its availability is tightly controlled by zinc homeostatic proteins such as importers, exporters, sensors, and metallothioneins.

Among the different redox zinc switches, we distinguish *redox sensors* and *redox transducers.* In redox sensors, zinc is released for the purpose of altering protein function without any apparent function for the released zinc ion. In redox transducers, zinc is released for a purpose such as binding to another protein and affecting its function, while conformational changes of the protein to which zinc was originally bound may or may not be important. Thus, redox zinc switches in proteins transduce redox signals into protein functions or a "zinc signal." It is critical to appreciate the frequency of zinc/cysteine interactions in proteins to gauge the possible impact of redox processes on the functions of zinc proteins. Vice versa, the involvement of zinc in so many cysteine interactions indicates a significant control of zinc over the cellular thiol/disulfide redox state. In essence, the static principles of how zinc organizes protein domains are enlarged by dynamic redox processes that change this organization with multiple consequences for biological functions including receiving and generating cellular signals.

C. Redox zinc switches in the control of protein function, protein conformation, and protein–protein interactions

From prokaryotes to eukaryotes, redox zinc switches are emerging as a general principle for regulation of protein function, controlling chaperone activity, binding interactions of proteins with other proteins or DNA, and enzymatic activity. Proteins with such switches serve rather diverse functions and one wonders whether the known examples forecast a much larger number of proteins that employ these or similar principles. Oxidation can either activate or inactivate a biological process.

Paracoccus denitrificans glutathione-dependent formaldehyde-activating enzyme (Gfa) catalyzes the formation of *S*-hydroxymethylglutathione, the first step in the detoxification of formaldehyde. The protein has two zinc/thiolate centers, a tetracoordinate structural ZnS_4 center and an apparently tricoordinate catalytic ZnS_3 center (158). The proposed mechanism involves mixed glutathione disulfide formation coupled with association and dissociation of the catalytic zinc ion. Subsequently, *S*-hydroxymethylglutathione is oxidized by class III alcohol dehydrogenase to *S*-formylglutathione, which is hydrolyzed to glutathione and formate by *S*-formylglutathione hydrolase. Alcohol dehydrogenases are zinc enzymes. Nitric oxide (NO) inhibits their enzymatic activity and releases zinc (64).

Escherichia coli Hsp33 is a molecular chaperone that is induced by the heat-shock-specific sigma factor σ^{32} (163). Oxidation of the cysteine ligands of the zinc site in Hsp33 results in the formation of two disulfides between the four cysteines, zinc release, and a large conformational change (71, 101). Induction of chaperone activity requires dimerization of the oxidized monomers; zinc binding to the reduced cysteines prevents the dimerization (72). The human cochaperone Hdj2 is a homologue of DnaJ with an interleaved double zinc motif. However, instead of activation, as in the case of Hsp33, hydrogen peroxide *inactivates* Hdj2 and releases zinc. Thioredoxin reduces the oxidized protein *in vivo* (41).

The *Streptomyces coelicolor* σ^R protein is a key transcriptional regulator of the oxidative stress response (163). Binding of the anti-sigma factor RsrA (regulator of sigma R) to the σ^R protein inhibits transcriptional activity. The Zn/S site in RsrA is a sensor of disulfide stress[2] (122). Oxidation of the cysteine ligands and disulfide bond formation in RsrA expels zinc and stabilizes a protein conformation that allows dissociation of the sigma factor. The ensuing transcriptional activation of genes that counteract the disulfide stress includes the thioredoxin/thioredoxin reductase system, which reduces oxidized RsrA and restores its capacity to bind the sigma factor.

The activation of the mammalian transcription factor NF-E2-related factor 2 (Nrf2) embodies the same principle. In the cytosol, Nrf2 is bound to the Kelch domain of Keap1, a zinc metalloprotein with cysteine ligands (51). Induction of phase II gene transcription involves reaction of oxidants/electrophiles with the cysteine ligands of Keap1, followed by zinc release and translocation of Nrf2 to the nucleus (51).

The Zn/S site in the p70 subunit of heterotrimeric human replication protein A (RPA, also known as single-stranded DNA binding protein (SSB)), though not essential for its single-stranded DNA-binding activity, confers redox dependence on both its interaction with DNA and its recognition of damaged DNA (167).

Betaine-homocysteine methyltransferase is one of the two enzymes that regenerate methionine from homocysteine in the methionine cycle. When the human enzyme is oxidatively inactivated, the catalytic zinc dissociates upon disulfide formation with two of the three cysteine ligands (54). This process is reversible. At present it is unclear whether it is physiologically relevant. Human porphobilinogen synthase is activated by reduction of a disulfide bond in the active site and migration of a zinc ion from a distal site to three cysteines in the active site (180).

[2] Disulfide stress is a condition of oxidative stress that generates disulfide bonds in proteins (5, 46).

The zinc finger motif in the monomer of cellulose synthase (CesA) is proposed to participate in oxidative dimerization of the enzyme (116). Zinc maintains the protein in its monomeric state, a functional potential opposite to zinc-induced oligomerization in other proteins.

The oxidative activation of matrix metalloproteinases (128), a family of enzymes with 24 mammalian members (222), is yet another example of the redox activity of zinc/cysteine coordination environments. In the zymogen of these enzymes, a thiolate group contributed by a cysteine side chain blocks the catalytic site by completing the $ZnSN_3$ coordination (190). Reaction of this Zn–S bond with glutathione disulfide (206) or other oxidants such as nitric oxide (73) activates the proteinase. This principle of oxidative activation also applies to the ADAM (a desintegrin and metalloproteinase) family of enzymes. Among the 29 mammalian ADAMs, 17 contain the sequence motif for a catalytic site of a metalloproteinase (214). TNFα-converting enzyme (TACE) is activated by nitrosylation of a critical cysteine ligand (228). This example illustrates the principle that zinc binding to a cysteine ligand docks an entire protein domain, and that breaking a single Zn–S bond results in large global change. It also underscores the fact that different molecular mechanisms for cysteine oxidation exist. Oxidation can result in the formation of either one or two disulfides. Modification of a single ligand can be accompanied by either zinc release, for example, formaldehyde-activating enzyme (see above), or release of only the ligand while zinc remains bound.

Oxidation of cysteine ligands also can affect the quaternary structure of proteins because zinc ions can serve as a crosslink between different protein chains. In such interfacial zinc sites, zinc is either essential for protein–protein interactions, the assembly of macromolecular complexes, or it is mainly a stabilizing factor (138). Zinc can crosslink four, three, or more commonly, two protein monomers (138). Functions of these interfacial zinc sites include catalysis, inhibition of enzymatic or other activities, packaging proteins for storage, formation of protein/receptor complexes, and construction of molecular scaffolds (138). Control of the availability of zinc and cysteine ligand-centered redox reactions in interfacial zinc sites suggest mechanisms for modulating transient protein–protein interactions. In the nitric oxide synthase dimer, oxidation of the thiolate ligands of the zinc ion that bridges the subunits releases the zinc ion. As a consequence, the dimer dissociates into inactive monomers, thereby uncoupling nitric oxide synthesis (230). Such dissociation can be effected by *S*-nitrosation of the cysteine ligands and disulfide formation in inducible nitric oxide synthase, suggesting both a possible mode of inhibition and regulation of the monomer–dimer equilibrium (120, 153).

In addition to functioning as redox sensors, zinc/thiolate sites can be zinc sensors (18). The "zinc link", a zinc/tetrathiolate that organizes the quaternary structure of the endoribonuclease RNase E, is such a potential zinc sensor. It was suggested that the catalytically active RNase E tetramer would not form under conditions of limited zinc availability, thereby affecting the stability of particular messenger RNAs (29).

There is the potential for zinc and redox modulation of heterologous protein–protein interaction (quinary structure) in an even larger number of proteins. A zinc dimerization motif with tetrathiolate coordination, termed a "zinc hook", holds together the coiled coils of the ATPase Rad50, providing a scaffold for association of the nuclease Mre11 with DNA (89). A similar zinc motif, termed a "zinc clasp", is essential for the association between the tyrosine kinase Lck ($p56^{lck}$) and the T-cell co-receptors CD4 and CD8a (106). It is unknown how these interactions are controlled. Regulation of the sulfur oxidation state of cysteine ligands by cellular zinc and redox control of zinc ion availability make it almost certain that such interactions are modulated by zinc and/or redox.

Summaries of these redox zinc switches, their functions, and their underlying redox chemistry are given (Table 1, Fig. 6).

D. Redox sensors and transducers in cellular signaling

While these examples demonstrate how redox sensing and transient zinc binding influence diverse proteins functions, the effect of redox on proteins with zinc/cysteine coordination environments in signaling cascades underscores more fundamental principles in cellular function and communication. Redox effects are expressed at several levels in a cascade, namely at the receptor through redox modulation of matrix metalloproteinases that proteolytically process receptors, at the phosphorylation state of proteins, at protein–protein interactions, and at transcription factor activity. Considering the large number of zinc finger proteins, one wonders how many of them are affected by redox signals under physiological conditions. Under conditions of oxidative stress, the function of a much greater number of proteins will be compromised. Research has been limited by the availability of purified zinc proteins and tools to investigate redox-mediated zinc release in cultured cells. In a few cases, it was possible to investigate the redox chemistry of the isolated proteins and to establish the redox sensitivity of particular zinc finger domains in tissue extracts. Demonstration that sulfur ligands are oxidized and release zinc *in vivo* continues to be experimen-

TABLE 1. EXAMPLES OF REDOX ZINC SWITCHES AND THEIR FUNCTIONS IN PROTEINS

Protein	*Control of Function*
Metallothionein	Zinc redistribution
Nitric oxide synthase	Subunit interaction
Anti-sigma factor RsrA	Disulfide stress sensor
Replication protein A	DNA binding
Betaine-homocysteine methyltransferase	Catalysis
Heat shock protein Hsp33	Molecular chaperone
Protein kinase C	Catalysis
Keap 1	Sensor for inducers of phase 2 response, activation of transcription factor
Formaldehyde-activating enzyme	Catalysis

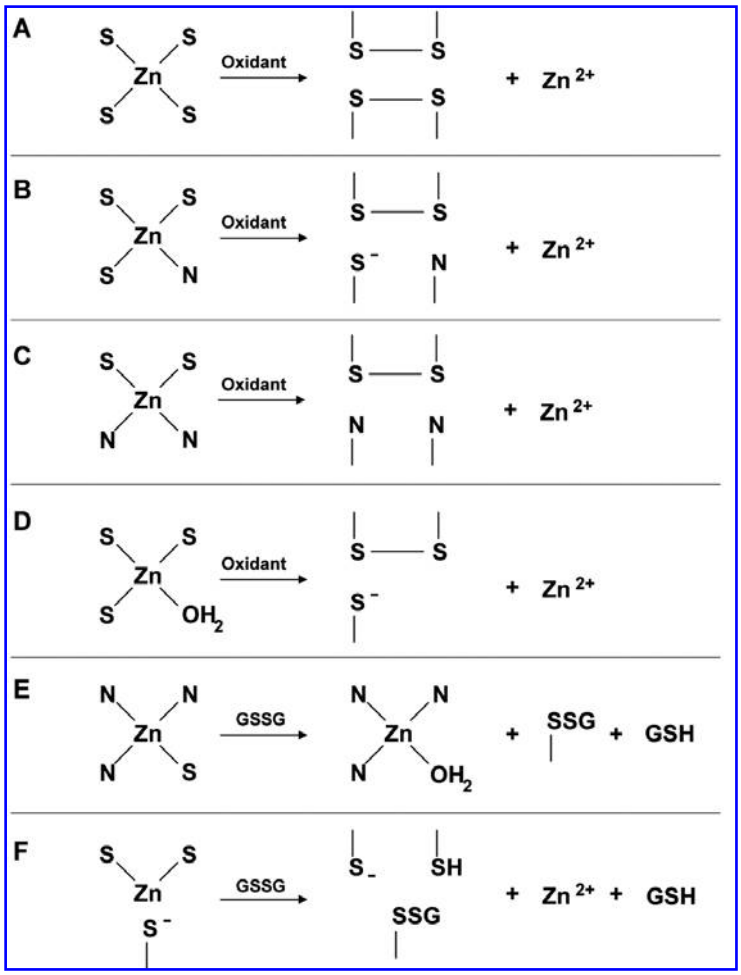

FIG. 6. Redox zinc switches in proteins. The first three examples illustrate the redox chemistry of structural Zn/S sites. (**A**) Oxidation with the formation of two disulfides bonds. In the *Escherichia coli* heat shock protein Hsp33, dimerization of the oxidized monomers induces molecular chaperone activity. Variations on the theme: In cellulose synthase, zinc keeps the protein in the monomeric state; in nitric oxide synthase, zinc binds at the dimer interface. (**B** and **C**) Oxidation with the formation of one disulfide bond such as in the *Streptomyces coelicolor* anti-sigma factor RsrA and in other zinc finger proteins. The remaining three examples illustrate the redox chemistry of catalytic Zn/S sites. (**D**) Disulfide bond formation in the catalytic site of human betaine-homocysteine methyltransferase inactivates the enzyme. In other enzymes such as human porphobilinogen synthase, reduction of a disulfide bond in the active site and zinc insertion may activate the enzyme. (**E**) Glutathione disulfide (GSSG) glutathionylates the cysteine ligand in the zymogen form of mammalian matrix metalloproteinases and activates the enzymes. Strictly speaking, this example does not represent a redox zinc switch as zinc does not dissociate from the enzymes. It merely illustrates the ligand-centered redox chemistry of another Zn/S site. (**F**) Glutathione disulfide also participates in the catalytic cycle proposed for the *Paracoccus denitrificans* glutathione-dependent formaldehyde-activating enzyme. For simplicity, the chemical reactions are not balanced. S, sulfur ligand from cysteine; N, nitrogen ligand from histidine. See text for details.

tally challenging, in particular for low abundance proteins. However, some technical limitations have been overcome by using commercially available, highly sensitive fluorescent chelating agents and fluorimetric techniques for *in situ* and *in vivo* imaging of picomolar zinc fluxes.

A recent forum on "Redox Control of Zinc Finger Proteins" in this journal provides an account of the state of the field up to the year 2000 (119). The following examples will update this account.

a. Transcription factors. The nuclear receptor superfamily contains four classes of proteins: steroid receptors, retinoid X receptor (RXR) heterodimers, dimeric orphan receptors, and monomeric/tethered orphan receptors (159). The nuclear hormone receptors transduce signals from glucocorticoids, mineralocorticoids, sex steroids (estrogen, progesterone, testosterone), thyroid hormones, and vitamin D_3. The family also includes receptors for retinoic acid, fatty acids, prostaglandin derivatives, and other ligands (peroxisome proliferator-activated receptors). Most of the members of this receptor family have two zinc ions in their DNA-binding domain. In the estrogen receptor, the zinc finger that mediates receptor dimerization, but not the one involved in DNA binding, is preferentially oxidized, precluding dimerization and abrogating DNA binding (215). Cysteines in the ZnS_4 coordination environments are not chemically equivalent as their reactivity is differentially modulated by nearby basic amino acids (7). Nitric oxide reacts with the cysteines coordinating zinc in the estrogen receptor, inhibiting their DNA-binding (62). The transcriptional activity of the vitamin D_3 and retinoid X receptors are inhibited by nitric oxide (115). *In vitro* data demonstrate that oxidation of the sulfur ligand in the retinoic acid receptor (RARα) leads to zinc extrusion and loss of DNA-binding activity (30).

The tumor suppressor p53 controls the production of reactive oxygen species, initiates apoptosis, and inhibits the cell cycle via induction of the inhibitor p21. A zinc ion in an S_3N coordination environment is essential for maintaining the structure of p53 and for its interaction with DNA (39). Its DNA-binding activity is affected by changes of either the concentrations of zinc or the cellular redox state. The redox sensitivity of p53 is thought to be a biochemical mechanism by which p53 can serve as a sensor of multiple forms of stress (80).

The RING (really interesting new gene) finger domain is yet another zinc-binding motif frequently found in E3 ubiquitin ligases. The RING finger of the APC11 subunit (anaphase-promoting complex) releases zinc when treated with hydrogen peroxide, suggesting that a peroxide signal

contributes to the delay in progression of the mitotic cell cycle under oxidative stress (31).

b. Signaling enzymes. The cysteine-rich C1 domain of protein kinase C (PKC) isoforms binds two zinc ions in a double zinc motif with a set of two S_3N ligands. In addition to its activation by diacylglycerol, PKC is activated by oxidation (69). Superoxide stimulates PKC activity by oxidizing the thiols in the cysteine-rich region and releasing zinc (110). Hydrogen peroxide and phorbol esters release zinc from an isolated PKC fragment, from a PKC immunoprecipitate, and in single cells (113), demonstrating that redox and lipid signals converge at the Zn/S centers. The roles of zinc are described as linchpins that are pulled in the process of activating PKC. Phorbol ester-induced zinc release may involve destabilization of a histidine ligand of zinc. Oxidants and lipids act on different zinc ions because when both are applied together, two zinc ions are released. cRaf has similar Zn/S sites and is also activated by oxidant-induced zinc release. Both PKC and cRaf contain binding sites with nanomolar affinity for retinol (90). A vitamin A-dependent signaling pathway also converges at the zinc finger domain and enhances the redox-mediated activation of these enzymes.

In addition to serine/threonine kinases, zinc and redox affect phosphorylation signaling of protein tyrosine phosphatases. The catalytic cysteine of these enzymes is inactivated by oxidation and formation of a sulfenyl amide intermediate between the sulfur atom of Cys-215 and the main chain amide of the neighboring Ser-216 (179). These enzymes do not contain zinc in their active sites. However, their activity is inhibited by low nanomolar concentrations of zinc (75, 144). They seem to be modulated by cellular zinc because addition of chelating agents to cultured cells changes the phosphorylation state of their substrates (75). Zinc binding has been mapped to the catalytic subunit of the protein tyrosine phosphatase SHP-1 (78). Zinc may protect the catalytic cysteine from oxidative inactivation or modulate the activity of protein tyrosine phosphatases in addition to or in combination with redox regulation.

E. Chemistry of zinc/thiol(ate) oxidation

Zinc binding changes the properties of the thiol group in cysteine. One aspect is a lowering of the pK_a value of the thiol/thiolate couple, allowing the nucleophilicity of thiolate to be utilized at physiological pH. This change of reactivity is differentially expressed in the different coordination motifs. In alkylations, a zinc center with four cysteines has higher reactivity than one with three or two cysteines (216). Hydrogen bonding (NH $\cdots$ S) drastically influences the reactivity of the sulfurs (38, 186). The dielectric properties of the protein and electrostatic screening of zinc sites also affect reactivity as does the redox potential of the Zn/S site (148, 201). Because entropic factors determine the redox potential of thiol/disulfide pairs the zinc ion is an important determinant of the redox potential. Zinc aligns the thiol groups and establishes their proximity. The redox potential remains so low that mild cellular oxidants such as glutathione disulfide react with Zn/S sites and release zinc (131). This property makes Zn/S sites a target of redox signaling. Nitric oxide (NO) has received considerable attention as a signaling molecule that reacts with the cysteine ligands in zinc finger proteins. Signaling by other reactive species such as peroxide, superoxide, and sulfur and selenium compounds is similarly effective. It is unknown whether a single species is primarily responsible for oxidizing Zn/S sites under physiological conditions. Under oxidative stress, the chemistries of reactive oxygen, nitrogen, and sulfur species are all relevant as these oxidants target Zn/S sites. Last but not least, toxic compounds and drugs that are not necessarily oxidants but electrophiles can react with Zn/S centers and release zinc with critical effects on protein function and zinc metabolism.

The formal oxidation state of sulfur in cysteine is -2 (RSH). In biology, the reversible oxidation states of sulfur are the disulfide (-1) (RSSR), the sulfenic acid (0) (RSOH), and the sulfinic acid (+2) (RSO_2H). A host of enzymes reduces sulfur in higher oxidation states in these species to thiol sulfur. For example, sulfiredoxins reduce cysteine sulfinic acid, a sulfur oxidation state that together with the oxidation state of sulfur in sulfonic acid (+4) (RSO_3H) was believed to be irreversible in biology (32). Sulfur in cysteine also participates in radical reactions forming thiyl radicals.

Disulfides are the only oxidation states identified so far as the result of sulfur ligand oxidation in Zn–S sites. The fact that there is no information about higher oxidation states of sulfur during oxidation of zinc-bound cysteines should not be taken as evidence that they are not formed. Disulfide S–oxide formation was observed when a peptide corresponding to a Sp1 zinc finger and containing nickel instead of zinc was treated with oxygen (219). In model complexes of ZnS_2N_2 coordination environments, ligands were oxidized to disulfonates, in most cases followed by zinc release (2). Clearly, more work is needed to address the chemistry of ligand-centered oxidation and to understand reactions leading to higher sulfur oxidation states and irreversible damage of ligands under conditions of high oxidative stress and oxidation by specific xenobiotics.

a. Reactive species. Nitric oxide (NO), superoxide and peroxide are the main reactive species discussed in redox signaling. The reaction of Zn/S sites with nitric oxide can lead to *S*-nitrosylation and disulfide formation (114). Another reaction is transnitrosation, a transfer of NO from *S*-nitrosothiols to an acceptor (36). NO also reacts with superoxide to form peroxinitrite, a very powerful oxidant of Zn/S sites (22). Superoxide and peroxide also release zinc from proteins with zinc/thiolate coordination (110, 174).

b. Sulfur compounds. Disulfides release zinc from Zn/S sites by thiol/disulfide exchange reactions (131). In analogy to reactive oxygen species and reactive nitrogen species, the concept of reactive sulfur species was introduced (66, 67). Disulfide *S*-monooxides (thiosulfinates) and disulfide *S*-dioxides (thiosulfonates) are strong oxidizing agents toward thiol sulfur in proteins. These compounds are formed under oxidative stress (61, 121), release zinc from Zn/S sites, and form mixed disulfides and the corresponding acids.

c. Selenium compounds. Selenium is an essential trace element. In the form of selenocysteine (Sec), the "21st amino acid," selenium is incorporated cotranslationally into selenoproteins. As a homologue of sulfur in the periodic system of the elements, the biological chemistry of selenium bears some resemblance to that of sulfur. Biology capitalized on the differences between sulfur and selenium, in particular the significantly lower pK_a value of the selenol/selenolate couple than that of the thiol/thiolate couple, and the different redox potentials of selenium oxidation states as compared to those of sulfur. Like sulfur in biology, selenium undergoes redox reactions, and hence is utilized as a cellular oxidant toward thiols. In the selenium enzyme glutathione peroxidase, for example, peroxide oxidizes the selenol group of the active site selenocysteine to the corresponding selenenic acid, which then oxidizes the thiol group of glutathione to form glutathione disulfide. Remarkably, this biological chemistry of selenium extends to cysteine ligands of zinc (100). Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one), a selenium-containing redox drug and a glutathione peroxidase mimic (184), releases zinc from the zinc protein metallothionein (MT) within seconds. The reaction follows a 1:1 stoichiometry with respect to thiols in MT and takes place even in the presence of an excess of glutathione (98). Selenocystamine reacts with MT at much lower concentrations than cystamine (100, 132). Low molecular weight reducible selenium compounds oxidize MT or reduce the oxidized protein to generate the apoprotein thionein (T) (34, 35). Reactions resulting in zinc release occur at substoichiometric amounts of the selenium compound with regard to thiols, demonstrating a *catalytic* action of some selenium compounds (34, 35). Moreover, selenium compounds catalyze either the release of zinc from MT or the reduction of oxidized T (T_O) by promoting tight coupling with the glutathione (GSH)/glutathione disulfide (GSSG) pair (Fig. 7). Released zinc induces the expression of T, whose 20 cysteines contribute to the cellular redox buffering capacity. In this way, the two essential elements zinc and selenium interact and antioxidant effects of selenium compounds are expressed through oxidative chemistry on sulfur ligands in zinc metabolism.

Redox reactions of selenium compounds are not restricted to MT. Zinc finger motifs are highly reactive towards oxidizing selenium compounds. Diselenides such as selenocystine or compounds with selenium in higher oxidation states release zinc from the zinc fingers in formamidopyrimidine-DNA glycosylase (FPG) and xeroderma pigmentosum group A protein (XPA), enzymes that are involved in base and nucleotide excision repair (20).

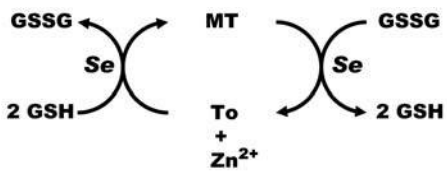

FIG. 7. Coupled metallothionein and glutathione redox cycles. Selenium redox catalysts increase the rates of both MT oxidation by glutathione disulfide (GSSG) and T_O reduction by glutathione (GSH). MT and T_O function like glutathione peroxidase and glutathione reductase, respectively, but noncatalytically.

These interactions between selenium compounds and Zn/S centers demonstrate zinc release by an oxidative reaction in a generally reducing environment. They occur even in the presence of a relatively large excess of reduced thiols such as glutathione. The catalytic potential of selenol(ate)s suggests that modulation of zinc release and binding are significant aspects of selenium's pharmacology in cancer prevention, diabetes, and anti-inflammatory therapy. This potential of restoring redox cycles has been demonstrated by preventing increases of cellular available "free"[3] zinc and decreases of both MT and glutathione levels in diabetic rats that received injections of sodium selenite (11).

F. Cellular redox changes and redox compartmentation

Tenets of redox signaling are that (a) oxidative mechanisms of activation can occur in an overall reducing environment such as the cytoplasm, (b) different redox couples are held at different redox potentials and are not in equilibrium, and (c) there is redox compartmentation within the cell. The redox potentials of proliferating and confluent fibroblasts differ by 34 mV (91). Redox potentials of the GSH/GSSG couple change from –240 mV in proliferation to −200 mV in differentiation to −170 mV in apoptosis (181). Differentiating and apopotic cells have redox potentials that are 60 and 72 mV more oxidizing with regard to the redox potential of normal cells (28, 109). A change of 60 mV corresponds to a 100-fold change in the GSH/GSSG ratio. Such a change is conducive to alter the zinc content of proteins with zinc/cysteine coordination (94). Moreover, the GSH/GSSG couple is held at −239 mV in the cytosol, whereas more oxidizing conditions prevail in the endoplasmic reticulum where the redox potential of that couple is −165 mV (92). Other oxidative environments are the mitochondrial intermembrane space (IMS), endosomes, and lysosomes (10). The mitochondrial IMS allows oxidative protein folding that controls the zinc-binding of proteins with Zn/S sites (see below). The IMS is believed not to be in redox equilibrium with either the cytosol or the mitochondrial matrix (82). Perhaps a similar situation pertains in the endoplasmic reticulum, where the cysteines of protein disulfide isomerase may bind zinc (188). Thus, redox compartmentation within the cell as well as redox changes in the cytosol might be critical determinants of the metal-binding capacity of proteins with Zn/S sites and the availability of zinc in different compartments.

[3] Cellular available "free" zinc refers to the amount of zinc that is freely and readily available to a chelating agent. Other terms used in the literature are "labile" or "readily exchangeable" zinc. For the lack of a better term, "free" zinc ($[Zn^{2+}]_i$) is used in this article, albeit with the understanding that the ion is not devoid of any ligands.

III. METALLOTHIONEIN AND THIONEIN: A LINK BETWEEN REDOX AND ZINC SIGNALING

Metallothioneins (MT) are major cellular transition metal ion-binding proteins in cells. They were the first proteins known to have zinc–sulfur coordination bonds. Their Zn/S sites have served as a paradigm for zinc transfer between proteins, redox functions of zinc proteins, and the link between redox and zinc metabolism (99, 131, 133, 134, 136, 137, 142). MTs are assigned to three classes (104). The proteins in class I have strong homology to mammalian MTs. Class II MTs are a heterogeneous group of proteins with high thiol content, metal binding characteristics, and some primary structural features that are reminiscent of those of class I MTs. Cysteine-containing peptides and glutathione analogues such as phytochelatins belong to class III. In humans, more than a dozen genes encode a family of class I proteins with 60–68 amino acids (MT-1 and related proteins, MT-2, MT-3, and MT-4). The name metallothionein derives from the observation that the protein contained a relatively high amount of sulfur and several metal ions, notably cadmium, when it was first isolated from horse kidney (130). The finding of cadmium in a protein stimulated interest and research in a possible role in detoxification of this toxic and carcinogenic metal. Cadmium is a congener of zinc and accumulates with age, in particular in the kidney. Under most physiological conditions MT contains zinc, and therefore, the following discussion will focus on zinc-containing MT.

Mammalian MTs are two-domain proteins. The protein completely envelops the seven zinc ions that are bound by twenty cysteines in two zinc/thiolate clusters (Fig. 8), one with three zinc ions and nine cysteines in the N-terminal domain, and the other with four zinc ions and eleven cysteines in the C-terminal domain. In contrast to the zinc ions, which are buried in the protein, some of the sulfur ligands are accessible at the surface of MT (176). Owing to these cysteines, MT is a relatively reactive molecule both *in vitro* and *in vivo*, and a prime example of a redox-active zinc protein (131, 134, 136). Oxidation of its thiolate ligands and concomitant zinc release provide a mechanism to explain how zinc can be tightly bound to protect the cell from the deleterious effects of the free zinc ion (see below in section IVBb) and still be available for cellular processes (143). This ligand-centered redox chemistry also can release cadmium and copper when these metal ions bind to MT under specific conditions (125, 152).

Remarkably, mild biological oxidants such as glutathione disulfide (GSSG) release zinc from MT, indicating a low redox potential of MT and raising the possibility that oxidation is a physiological mechanism for zinc release (131, 132). Reactions with GSSG and other disulfides are slow, but can be enhanced by additional factors such as selenium compounds that either react directly with MT or catalyze the oxidation of MT by disulfides (35, 100). Oxidants, including reactive species, release zinc in cellular systems. Selenite, membrane-permeable disulfides, and hydrogen peroxide release zinc in cardiomyocytes, neurons, and promyelocytic leukemia cells, respectively (1, 174, 205). While many oxidants release zinc under oxidative stress, it is unknown whether a single agent, multiple redox pairs, or enzymes react with MT and release zinc under physiological conditions. The recognition of this redox chemistry of MT led to the idea that MT is a transducer of redox signals into "zinc signals" (Fig. 9, top). MT is a target of nitric oxide (189, 192). Nitric oxide or its adduct with a thiol, *S*-nitrosothiol, releases zinc from MT *in vitro* and in cultured cells (15, 114, 168).

S-nitrosothiols react preferentially with the isoform MT-3 by transnitrosation, a process in which NO transfer occurs between sulfhydryl groups (36). This preference is caused by two cysteines that are flanked by consensus motifs for catalytic nitrosylation in the primary sequence of MT-3. (K)C(E) and C(E) motifs are present only in MT-3 and not in the MT-1, MT-2, or MT-4 isoforms, rendering MT-3 particularly sensitive to nitric oxide but not to peroxide. The isoform MT-3 is a neuronal growth inhibitory factor (GIF) in the brain (207), a biological activity that it does not share with other MT isoforms. MT-3 has been isolated on the basis of the fact that its growth-inhibitory activity on rat embryonic cortical neurons is absent in extracts from Alzheimer disease brains (207). It undergoes a redox transition that converts its 4-zinc cluster to a 3-zinc cluster (177). Based on the observation that MT-3 occurs in approximately equal amounts inside and outside the cell (208), it has been suggested that the 3-zinc and the 4-zinc cluster constitute its extracellular and intracellular forms, re-

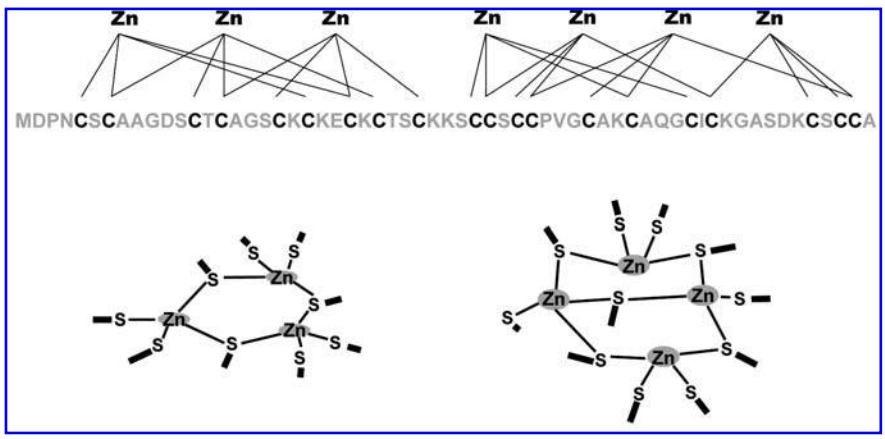

FIG. 8. Metallothionein primary sequence (human MT-2), zinc binding, and the structures of the two zinc/thiolate clusters. *Left*: Zn_3Cys_9 cluster in the N-terminal domain. *Right*: Zn_4Cys_{11} cluster in the C-terminal domain.

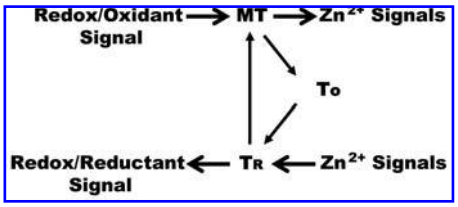

FIG. 9. Metallothionein and thionein as transducers of redox and zinc signals. The redox zinc switch in MT transduces a redox (oxidant) signal into a zinc signal with different specificity. In this process, oxidized thionein (T_O) is formed. Zinc induces the expression of reduced thionein (T_R), which has twenty thiols. Therefore, a zinc signal also can be transduced into a redox (reductant) signal.

spectively (177). MT-3 binds to the GDP complex of Rab3A, a small GTPase that interacts with docking and trafficking proteins in the exo-endocytotic cycle of synaptic vesicles, and is thought to be involved in the pathway of loading synaptic vesicles with zinc (111). Synaptic zinc vesicles colocalize with some glutamate vesicles and release their zinc into the synaptic cleft when the nerve is stimulated (59, 60).

A. Speciation and redox biology of metallothionein and thionein

While these investigations show that MT can be oxidized with concomitant zinc release, they have not clarified the fate of the protein in this process. Since isolations of MTs are based on their metal content, the apoprotein would escape detection. A new method based on differential chemical modification of cysteines in their metal-bound and free form made it possible to distinguish the holoprotein (MT) from the metal-free apoprotein thionein (T) (220). In the liver, kidney, and brain of rats, the amounts of T are commensurate with those of MT. Information on whether T is fully reduced or partially oxidized is not available from this assay because it must be performed in the presence of a reducing agent. Oxidized protein (thionin) can now be analyzed with double differential modification assays in the presence and absence of either a chelating agent or a reducing agent. In this way, both the zinc-load and the redox state of the protein can be determined (76). Disulfide species of "MT" were detected in murine hearts that overexpress MT (55). The amount of oxidized protein increases when the heart is oxidatively stressed with the anticancer drug doxorubicin. A unique structure of the oxidized protein was not found, however, nor was there any evidence for disulfides formed in the N-terminal domain of MT. Twenty cysteines potentially form over 650 million species with different intramolecular disulfide bonds (97). To complicate matters further, MT can also engage in intermolecular disulfide formation. An intermolecular disulfide links the C-terminal domains of two MT-1 molecules (225). Sequence motifs such as Cys–Cys and Cys–X–X–Cys in MT and the orientation of cysteines by zinc in the clusters are expected to limit the number of possible disulfides. The cluster structures may contain a yet to be deciphered blueprint for the formation of specific disulfides and pathways of zinc release (143).

In summary, MT is not just one species but exists in different states depending on the redox environment. Potentially, different redox pairs and enzymes are involved in interconversion of these species. For example, T is an excellent substrate for a member of the flavin-dependent quiescin–sulfhydryl oxidase family of enzymes that participate in disulfide formation in oxidative protein folding (76, 199). The oxidized protein can be reduced with glutathione in the presence of a selenium catalyst (35). Thiol/disulfide oxidoreductases such as thioredoxin/thioredoxin reductase may also reduce the oxidized protein. The reversibility of these reactions *in vitro* suggests a redox cycle of MT dependent on other cellular redox couples such as glutathione/glutathione disulfide (GSH/GSSG) and linked to the control of cellular available "free" zinc (35).

Another important aspect of this redox cycle is that zinc ions induce the synthesis of glutathione and T, also through their activation of MTF-1. Induction of T serves at least two purposes. T is a chelating agent that can bind the excess of released zinc, thus stopping the zinc signal. Owing to its twenty reduced cysteines, T (T_R) is also a very efficient reductant. Therefore, in this redox cycle, zinc signals are also transduced into redox signals (Fig. 9, bottom).

B. Participation of metallothionein and thionein in signaling pathways

The view emerges from cellular studies that the MT redox cycle is a component of NO signaling and other cellular signaling pathways. The activation of muscarinic receptors or calcium influx into sheep pulmonary artery endothelial cells results in a FRET (fluorescence resonance energy transfer) signal of a GFP-based MT sensor (168). The sequence of events includes the calcium activation of endothelial nitric oxide synthase, NO production, and NO-induced zinc release from MT. Similarly, activation of murine aortic endothelial cells with pro-inflammatory cytokines releases zinc in the nucleus through activation of inducible nitric oxide synthase and nuclear translocation of MT (189).

The extensive transcriptional, posttranscriptional, and epigenetic regulation of MT-1/-2 gene expression demonstrates a high degree of integration into cellular signaling and is consistent with a role of MT in development and in all aspects of cell fate—proliferation, differentiation, and apoptosis. The levels of MT are highly variable; there is an over 400-fold variation of its total amount through isoform- and tissue-specific expression (217). Until now, functions of the induced or overexpressed protein had been discussed as properties of MT. It is, however, by no means a foregone conclusion that MT forms concurrently with T on the ribosome because zinc is not necessarily readily available. Therefore, many, if not all of the functions of these inducers could relate to the properties of T as a zinc acceptor (chelating agent) and reductant—a view opposed to the one commonly expressed. The extensive list of inducers includes metals, various types of stress, and acute phase and inflammatory responses (103). The promoters contain MREs, E box motif for binding of upstream stimulatory factor (USF), antioxidant response element (ARE), GC box for Sp1 binding, enhancer elements for activator protein-1 and -2 (AP-1/-2), and glucocorticoid response ele-

ments (84). Interferons, cytokines, growth factors, and agents that act on nuclear hormone receptors bind to specific cis-acting elements of the T genes and induce the protein. Oxidative stress induces T directly through action on the ARE and indirectly through MTF-1. Not only are many transcription factors zinc proteins (Sp1, nuclear hormone receptors, MTF-1) (Fig. 10), but zinc is also a constituent of the zinc finger protein PZ120, a transcriptional repressor (196).

Ever since its discovery (130), the function of MT has been shrouded in uncertainty and there have been competing views about its function (105, 165). The chemical properties of MT and T as zinc donor and acceptor, respectively, their redox properties, and their cellular translocations provide a general framework for their biological activities. Jointly, these properties suggest that the MT/T pair serves a physiological function at a central node in the cellular signaling network where redox signals are transduced into "zinc signals" (Fig. 9). Mitochondrial shuttling (221), export of MT into the extracellular space (154, 202) and its translocation into the nucleus (37, 160) suggest a more general role in directing the flow of zinc across cellular membranes. The nucleocytoplasmic shuttling of MT requires energy (ATP), depends on signaling kinases, the state of the cell, and oxidation of a cytosolic factor (4, 182, 197, 218).

It remains to be shown whether any or all of these translocations relate to the interaction of MT with ATP or GTP (95). ATP changes the shape of MT from that of a dumbbell to one that is slightly bent by about 20 degrees around the central hinge region that links the domains (146). Such conformational changes could determine its cellular translocation or retention in a particular compartment.

In conclusion, MT translocates to different compartments and buffers zinc in a process with high integration into cellular signaling pathways. It transduces redox signals. MT donates zinc and T accepts zinc. Because of these redox- and zinc-dependent characteristics, MT has been implicated in numerous cellular functions.

C. Released zinc ions as signals

The consequences of oxidant-released zinc from proteins are twofold. First, zinc compromises the function of the protein(s) from which it is released, and second, it affects functions of the proteins to which it subsequently binds (139). An important aspect is that zinc ions are now recognized as intracellular and intercellular messengers, which may rival calcium in significance (58). The concentrations of cellular available "free" zinc is in the picomolar/nanomolar range (14, 169, 185), though variations in mammalian cells are not known with great precision. The absolute basal levels measured are 25 p*M* for erythrocytes (185), 5 p*M* for pheochromocytoma (PC12) cells (198), 520 p*M* in cardiomyocytes (11), and 170 and 350 p*M* for monocytes and lymphocytes, respectively (79). It is clear that these levels are at least five orders of magnitude lower than those of the overall cellular zinc concentrations, which are a few hundred micromolar (164). Low nanomolar zinc fluctuations can be induced by stimulation of excitable cells (6), DNA-damaging agents (187), cellular influx of zinc (40), mitochondrial zinc release (183), and oxidative release of zinc from proteins when cells are exposed to oxidizing agents or when an oxidant signal is generated within the cell (1, 189, 192, 205). Because zinc fluctuations occur at such low concentrations and zinc interacts strongly with proteins, released zinc is a very potent signal. In signal transduction from a redox signal, a zinc signal with a different degree of specificity and selectivity is generated. Selectivity is determined by the characteristics of coordination sites in proteins. Whereas the coordination of zinc is generally flexible, certain constellations of a minimum number of ligands must be fulfilled to provide sufficiently tight binding. In the nanomolar range, zinc extends the signaling capabilities of calcium ions in the micromolar range and those of magnesium ions in the millimolar range (135). Thus, with these three redox-inert metal ions, the cell has a dynamic range of well over six orders of magnitude of concentrations for cation signaling. Multiple lines of investigations have been pursued to determine the target(s) of released zinc and the processes that are regulated by zinc ions. The consensus building from these studies is that zinc affects intermediary and energy metabolism, signaling, mitochondrial function, and gene expression.

a. Gene expression. MTF-1 (metal response element (MRE)-binding transcription factor-1) contains six zinc fingers arranged in tandem for interaction with DNA (3, 65). It is a zinc sensor and essential for basal as well as metal-induced expression of MT (88), and has served as a prime example of metal-induced eukaryotic gene regulation. Sensing involves

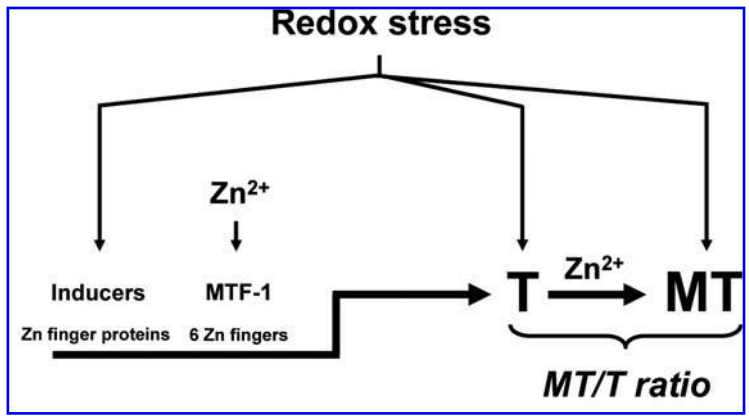

FIG. 10. Metallothionein gene regulation is redox- and zinc-dependent. Redox affects the state of the MT/T protein and gene expression of T. Gene regulation involves the zinc sensor MTF-1 and zinc proteins such as the transcription factors Sp1 and nuclear hormone receptors. At the protein level, the redox dependence of zinc-binding establishes a feedback loop: Induction of T restores reducing and zinc-binding capacity.

multiple fingers, responsiveness to an about 100-fold change in zinc concentrations, and binding to different types of MREs at low and high zinc concentrations (171, 213). A string of four closely spaced cysteines far from the zinc finger domain is also required for zinc-induced transcriptional activation (33). MTF-1 is constitutively expressed and its genetic ablation (knockout) is lethal at the embryonic stage (74). The activation is quite specific for zinc (19). MTF-1 is involved in the regulation of the stress response, including the expression of the heavy chain of γ-GCS (gamma-glutamylcysteine synthase) and many other genes (123). Oxidative stress and hypoxia activate DNA-binding activity of MTF-1 and increase MT expression (47,156). The activity of MTF-1 is modulated by MT and it is believed that it is the oxidative release of zinc from MT or the displacement of zinc from MT by cadmium that leads to the activation of MTF-1 (226). In sheep pulmonary aortal endothelial cells, NO-induced zinc release from MT leads to nuclear translocation of MTF-1 (193). Taken together, the oxidative activation of transcription by both MTF-1 and Nrf-2 are linked through a pool of available zinc that is controlled by MT/T (Fig. 11). MTF-1 also contributes to hypoxia-inducible factor-1 (HIF-1) activation by a mechanism that may involve perturbation of oxygen-sensing by increased glutathione levels (157). In other words, oxidative signals enable a concerted antioxidant response that includes a feedback mechanism whereby T lowers the availability of zinc. The capacity to reduce oxidized Keap1 and T_O is restored by induction of T and other antioxidant enzymes, including the thioredoxin/thioredoxin reductase system, by MTF-1 and Nrf2.

Genomic approaches have provided additional insight into zinc-dependent gene regulation. An increase in cellular zinc by either nutritional supplementation in mice or addition to the medium of cultured cells results in the activation of a large number of genes (44, 155). A global screen of the murine thymic transcriptome for genes that are modulated by changes in dietary zinc did not reveal any modulation of zinc metalloenzymes or zinc finger transcription factors but identified a number of genes involved in T-cell development, heat shock proteins, and translation- and transcription-related factors (155). An investigation of the effects of zinc-deficient and zinc-supplemented media on gene expression of human THP-1 mononuclear cells found 1045 zinc-responsive genes (5% cytoskeleton, 8–11% nucleic acid binding, 4–6% apoptosis, 10–18% metabolism, 4–8% cell growth/development, 10–16% signal transduction, 9–12% immune/cytokine, and 33–41% uncharacterized) (44). When hepatic gene expression was examined under zinc deficiency, genes related to growth, lipid and xenobiotic metabolism, stress response, nitrogen metabolism, intracellular trafficking, and signal transduction were affected (200). The "zinc regulon" in zinc-deficient human colon cancer HT29 cells comprises at least 309 genes, 79 of which play a role in intermediary metabolism, 30 in signaling, 15 in cell cycle and growth control, 15 in vesicular trafficking, 13 in cell–cell interactions, 10 in the cytoskeleton, and 19 in transcriptional control (107). More than half of the proteins that responded to high zinc in these cells participate in ATP production and in the stress response (108). The responses of cells or animals to zinc are rather pleiotropic. Zinc affects almost all aspects of cellular function, suggesting an indirect mechanism of gene activation and/or activation through zinc-dependent transcription factors other than MTF-1. Variation of zinc in the diet or in a medium of cultured cells presumably has far more complex effects than cellular zinc signals that are generated by specific signaling pathways.

b. Mitochondria. MT has a significant effect on mitochondrial respiration (221). When imported into the intermembrane space (IMS) of liver mitochondria, MT releases zinc, which inhibits mitochondrial respiration (221). Similar to other nuclear-encoded zinc- or copper-binding proteins that lack a mitochondrial targeting sequence but share conserved cysteine motifs, MT apparently diffuses through the translocase of the outer membrane (TOM) (87). A mechanism proposed for the vectorial transport of these proteins includes a disulfide relay system that catalyzes import into the IMS (149). Central to this process is a reaction cycle of the protein Mia40 (Tim40), the

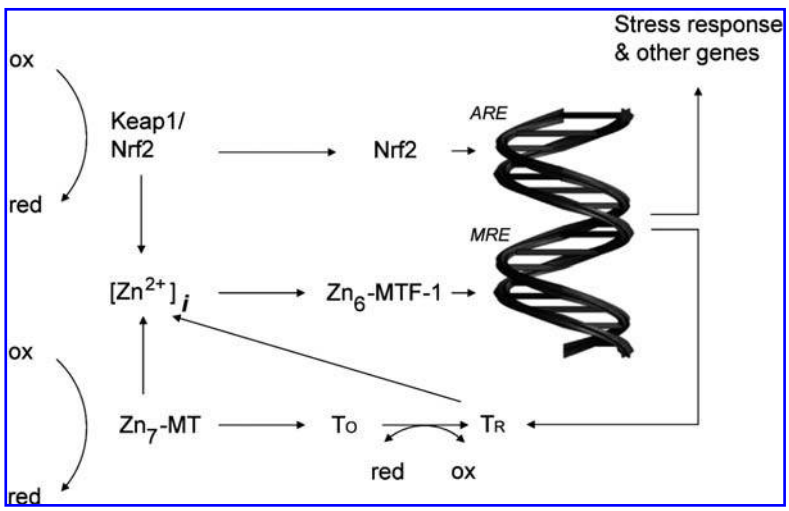

FIG. 11. Oxidant signaling in the parallel activation of both Nrf2- and MTF-1-dependent pathways. Two oxidative reactions increase the availability of zinc. Oxidation of Keap1 releases zinc, leading to the nuclear translocation of Nrf2 and its binding to the ARE (antioxidant response element) sequence. Oxidative zinc release from MT leads to activation and nuclear translocation of MTF-1 and binding to MRE (metal response element) sequences. It remains to be shown whether zinc release from Keap1 is sufficient for transcriptional activation of MTF-1. Both transcription factors participate in cellular redox homeostasis. The same or different redox pairs (ox/red) can release zinc from Keap1 and MT. A third redox pair can be coupled to the T_R/T_O equilibrium. With regard to zinc, the net effect of oxidative stress in this scheme is a stress response with biosynthesis of T (T_R) for controlling the availability of zinc.

sulfhydryl oxidase Erv1, and the protein to be imported. The cycle includes oxidation of Mia40 by Erv1, a transient mixed disulfide between the sulfhydryls of Mia40 and those of the imported protein, and thiol/disulfide interchange to generate the oxidized imported protein and reduced Mia40. The IMS is not in redox equilibrium with the cytosol and an oxidative environment for protein folding (126). In the IMS, zinc is removed from zinc proteins for oxidative folding, an essential step for formation of the TIM (translocase of the inner mitochondrial membrane) complex (127). Tim10, one of the small Tim proteins with twin Cys–X–X–X–Cys motifs (112), binds zinc in an S_4 motif; it has a low redox potential as GSH cannot reduce the oxidized protein, but GSSG can oxidize the reduced protein. Zinc maintains the protein in a reduced form for mitochondrial import. Since small Tim proteins apparently occur in both their disulfide and zinc-bound, reduced states in the IMS, the oxidatively folded protein must be either reduced for zinc binding or the zinc-bound form presents the protein before it reacted with Mia40. Oxidized small Tim proteins may be reduced by Hot13 (helper of Tim) (46). MT undergoes zinc–thiol/disulfide interchange (131). Therefore, these mechanisms provide a plausible explanation for how MT is imported into the IMS and releases zinc.

Complexes I and III of the respiratory chain have inhibitory, high-affinity (submicromolar) zinc binding sites (27, 124). In complex III, two zinc-binding sites have been identified (17). Inhibition of mitochondrial respiration is specific for the N-terminal β-domain of MT (221), which has a different zinc transfer potential than the C-terminal α-domain that is inactive in this regard (96). T, on the other hand, activates zinc- or MT-inhibited respiration. Also, state 3 (coupled) respiration increases when isolated mitochondria are treated with zinc chelating agents, indicating that normal respiration is modulated by zinc (221). Mitochondrial functions are particularly sensitive to zinc. Zinc inhibition of mitochondrial respiration increases the production of reactive oxygen species (ROS) (183). Nanomolar concentrations of zinc induce MPT (mitochondrial permeability transition) and inhibit the thiol oxidoreductase activity of lipoamide dehydrogenase with concomitant production of reactive oxygen species (63, 93). Zinc inhibits mitochondrial aconitase (43), which accepts zinc directly from MT (56). How and whether MT enters the mitochondrial matrix is unknown.

c. Zinc inhibition of enzymes. Nanomolar concentrations of zinc inhibit enzymes in energy metabolism and signaling (75, 144, 145). Enzymes in glycolysis and cytosolic glycerol 3-phosphate dehydrogenase are examples of such tightly inhibited enzymes (144, 145). These findings expand the repertoire of zinc functions because they identify zinc-dependent functions of proteins that are generally not considered to be zinc metalloproteins. Such tight zinc binding suggests specific structural requirements of the binding sites. Since zinc binding to these proteins has not been characterized structurally, it is unclear whether such potential regulatory zinc sites have a common motif.

While protein tyrosine phosphatases (PTPs) and caspase-3 were known to be inhibited by zinc (24, 170), more recent investigations demonstrated much stronger zinc inhibition at concentrations that can be achieved during oxidative zinc release. The IC_{50} for zinc inhibition of PTP 1B is 15 n*M*, and inhibition of other PTPs is similarly strong (75, 78). Among other targets, PTP 1B controls the phosphorylation state of the insulin receptor. Zinc inhibition of this enzyme could account for the insulinomimetic effects of zinc (77). Inhibition of PTP 1B maintains the insulin receptor in its phosphorylated state, which is necessary for propagation of the signal. The insulin signal generates a redox signal via coupling to the oxidase Nox4 (70). The resulting hydrogen peroxide either directly affects the activity of PTP 1B or releases zinc from MT for subsequent inhibition of PTP 1B. In any event, zinc inhibition of protein tyrosine phosphatases rather than zinc stimulation of tyrosine kinases could be the mechanism by which zinc modulates phosphorylation signaling (77).

Caspases are another enzyme family with a catalytic cysteine residue. Their activity is also affected by cysteine redox chemistry and by zinc inhibition. The IC_{50} for zinc inhibition of caspase-3 is <10 n*M* (144). Zinc might serve to reversibly bind and protect the essential catalytic cysteine from oxidative damage during activation of pro-caspase-3 (203).

IV. ZINC AND REDOX CONTROL

Beyond the molecular functions of redox zinc switches, there is an issue with even greater implications, namely to what extent zinc controls redox metabolism. It has been known for some time that zinc elicits antioxidant effects. However, it can also express oxidant effects when there is either too much zinc (overload) or not enough zinc (deficiency). Both conditions generate oxidative stress, albeit by different mechanisms (83). Tight control of cellular zinc by zinc homeostatic proteins is critical to maintain thiol/disulfide redox homeostasis. The remainder of this article will analyze these relationships that suggest that zinc has been chosen for the control of some aspects of redox metabolism because of its tight interactions with proteins and its redox-inert characteristics. Given an overall cellular zinc concentration of >200 μ*M* and binding to a considerable number of cellular thiols, it is not unexpected that zinc influences the cellular thiol/disulfide redox balance.

A. Antioxidant effects of zinc

Zinc is considered to be an antioxidant because it protects the cell against oxidative damage by interacting with cellular thiols, precluding their oxidative inactivation, and by competing with redox-active metal ions that produce reactive oxygen species (25, 172). Because zinc itself is redox-inert, any antioxidant function must be indirect. Therefore, we proposed the term "pro-antioxidant" (83) to describe functions of zinc restricted to a range of physiological and perhaps pharmacological concentrations (Fig. 12A). Pro-antioxidant functions of zinc are expressed through the activation of the metal response element (MRE)-binding transcription factor-1 (MTF-1), which induces proteins involved in the stress response such as T and the heavy chain of γ-glutamylcysteine synthase for glutathione synthesis.

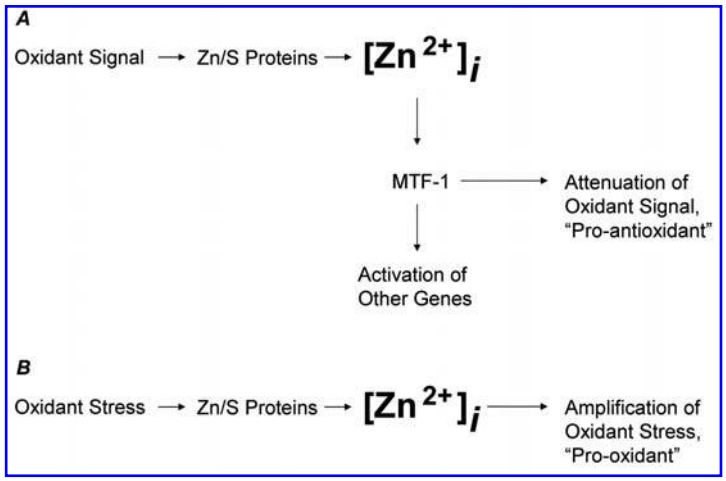

FIG. 12. Pathways for the expression of pro-oxidant and pro-antioxidant functions of zinc. The response to an oxidant signal can be either an amplification or an attenuation of the signal. (**A**) At physiological levels of oxidant signals, and perhaps pharmacological levels of zinc during zinc supplementation, zinc is a pro-antioxidant through its effects on gene expression. Zinc supplementation can provide protection, but it can also induce higher levels of MT and increase the amplitudes of zinc signals during oxidative stress. (**B**) Under oxidant (oxidative) stress, zinc released from zinc proteins binds to other proteins and elicits more oxidative stress. In the case of zinc deficiency, Zn/S sites might not be protected by zinc and hence susceptible to oxidation (not shown).

In humans and animals, zinc supplementation has antioxidant effects. When ten healthy humans received 45 mg/d zinc gluconate for 8 weeks, their inflammatory cytokine response was dampened (173). The authors suggest that an upregulation of A20, a protein with seven zinc fingers and a negative feedback regulator of NF-κB, is responsible for this effect. In human adults with type 2 diabetes, supplementation with 30 mg/day zinc over 6 months reduced the burden of oxidative stress by 15% (178).

Liver injury due to excessive alcohol consumption is thought to be mediated by oxidative stress. In a mouse model of alcoholic liver injury, zinc supplementation prevents alcoholic liver injury by inhibiting the generation of reactive oxygen species by cytochrome P450 2E1 and by enhancing the antioxidant response (229). Moreover, zinc supplementation prevented alcohol-induced myocardial fibrosis in the mouse model (212). In cultured cells, zinc has a pro-oxidant effect, but when the cells are pretreated with zinc, a *pro-antioxidant* effect against hydrogen-peroxide-induced cytotoxicity is observed (42). These antioxidant effects of zinc under physiological and pathological conditions suggest a therapeutic potential for zinc. However, the range between safe (recommended dietary allowances) and unsafe (reference dose) intake is relatively narrow. The borderline between pharmacological and toxic concentrations is ill defined. Hence, the margin for zinc supplementation is narrow because additional zinc can induce copper deficiency with severe consequences including oxidative stress (147).

Zinc elicits oxidative stress above and below its physiological and pharmacological concentrations (Fig. 12B). Strictly speaking, at low concentrations of zinc, it is its absence that has the pro-oxidant effect (161). These dual redox actions of zinc as either a pro-oxidant or a pro-antioxidant are dependent on the concentrations of cellular available "free" zinc and the zinc buffering capacity of the cellular components, which jointly determine whether zinc is cytotoxic or cytoprotective (Fig. 13). Within the range of operation of homeostatic mechanisms zinc attenuates the oxidant signal. If the concentrations of cellular available "free" zinc are too high, zinc amplifies the oxidant signal (Fig. 12).

B. Oxidant effects of zinc and zinc deficiency

a. Zinc deficiency and oxidative stress. Mammalian cells respond to zinc deficiency with a global change in gene expression (44, 48). How they sense zinc deficiency is unknown. Zinc deficiency induces oxidative stress by unknown mechanisms (86, 161, 162). One possibility is that transcription factors/signaling pathways sense oxidative stress rather than zinc deficiency.

b. Zinc overload and oxidative stress. If the capacity of the cellular zinc homeostatic system is overwhelmed, zinc overload elicits oxidative stress, depleting cellular energy (50) and increasing mitochondrial production of reactive species. These deleterious effects of zinc ions were studied initially in neurons, leading to the paradigm that intracellularly released zinc ions can be cytotoxic (59), despite the fact that zinc has very little acute toxicity systemically (209). Different mechanisms appear to underlie observations made in cultured cells and *in vivo*. Prolonged zinc supplementation elicits a conditioned copper deficiency that causes oxidative stress. Cellular zinc toxicity is evident when cells are exposed to concentrations of extracellular zinc that exceed the buffering capacity of the medium (usually >200 μ*M*). It remains unproven whether such studies are physiologically relevant because body zinc ho-

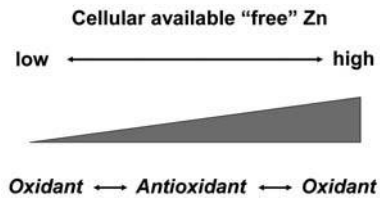

FIG. 13. Cellular zinc availability and redox functions. Tight control over the cellular available "free" zinc is necessary to control redox effects. Both low (deficiency) and high (overload) zinc elicit oxidative stress. Zinc expresses pro-antioxidant effects only in an intermediary range of physiological and possibly pharmacological concentrations.

meostasis apparently prevents zinc overload of cells. The main mechanism of increasing cellular available "free" zinc in both cellular studies and *in vivo* is the intracellular release of zinc by oxidative stress. Based on a large body of literature, the following sequence of events has been proposed to lead to neuronal death (22): calcium influx through calcium channels, calcium-activated nitric oxide production and peroxynitrite formation, zinc release from MT, zinc-induced production of reactive oxygen species by mitochondria, activation of the mitochondrial pathway of apoptosis, and a p38(MAPK)-mediated effect on potassium channels.

Depending on its zinc buffering capacity and plasticity in expressing defense proteins, the cell can cope with either too much or too little zinc to a certain degree, but initiates apoptosis if there is too much stress through either zinc deficiency or zinc overload (57, 195). The generation of oxidative stress is a plausible reason for the pro-apoptotic effects of zinc. Decrease of cellular zinc precedes even early markers of apoptosis (52). In a certain range, presumably where zinc has pro-antioxidant effects, zinc can also be anti-apoptotic. Inhibition of caspase-3 could be one reason for its anti-apoptotic effect. Pathways for modulation of cell death include the production of mitochondrial reactive species and the generation of pro-apoptotic signals through zinc proteins such as p53 and PKC. Other zinc-dependent proteins are IAPs (inhibitor of apoptosis proteins), which have zinc-containing BIR (Baculovirus IAP repeat) and/or RING domains (211).

Thus, another major theme is emerging, namely a role of the pool of cellular available "free" zinc in controlling the redox state and cell fate. Zinc- and redox-dependent signals converge in pathways that determine life and death of cells. The transcription factors NF-κB, p53, and AP-1 are major surveillance systems of cells. Their signaling is redox-regulated (194) and affected by the availability of cellular zinc (83). The way in which redox controls zinc and vice versa makes redox and zinc effects virtually inseparable, demanding that both be addressed experimentally to distinguish cause and effect.

V. DRUG INTERACTIONS

The relatively high chemical reactivity of Zn/S sites and the potency of the effects of released zinc have generated significant interest in drug discovery. The redox activity of Zn/S sites has been exploited for therapeutic purposes. Drugs containing a disulfide bond react with the cysteines in the ZnS_3N site of the nucleocapsid protein of retroviral viruses such as HIV-1, or the cysteine ligands of zinc in the human papillomavirus E6 protein (12), forming a mixed disulfide intermediate. Concomitant zinc ejection abolishes virus infectivity (175, 204).

Zinc-bound thiolate/disulfide interchange is also a mechanism of potent irreversible inhibition of β-lactamases with Zn/S sites and a possible strategy to combat antibiotic resistance due to expression of metallo-β-lactamases in bacterial infections (21).

Anticancer drugs such as chlorambucil and melphalan form covalent adducts with MT (223, 224). Rarely have the effects of these reactions on zinc homeostasis been considered, and it is in this area where there is a great therapeutic potential.

Motexafin gadolinium, in clinical trials for several cancers, mediates electron transfer from various cellular reductants including thiols, thereby generating hydrogen peroxide and superoxide (129). The drug increases cellular available "free" zinc and oxidative stress, both of which affect cancer cell growth and proliferation. These increases precede and correlate with cell cycle arrest and apoptosis (118). Cells treated with motexafin express MTF-1- and HIF-1 (hypoxia inducible factor 1)-regulated transcripts. Co-treatment with zinc and motexafin induces Nrf2-regulated transcripts.

VI. IMPLICATIONS FOR DISEASE

The implications of the basic chemical and biochemical mechanisms for the etiology of diseases, pathology, and the process of aging are too numerous to be discussed with reference to individual conditions. Zinc deficiency itself is a major risk factor for disease (209). Perturbation of zinc homeostasis should be considered as a critical component in any disease that is accompanied by oxidative/reductive stress. Oxidative cell injury occurs in exposure to drugs/toxic substances such as alcohol (ethanol), under conditions of inflammation and hypoxia, in diabetes, neurodegenerative diseases, cancer, coronary heart disease, viral infections, preeclampsia, asthma, cataracts, and yet other diseases.

VII. CONCLUDING REMARKS

A brief chronology of events highlights why the significance of zinc/cysteine interactions in proteins could only have been appreciated rather recently. Until the early 1980s, only two structurally characterized zinc sites with cysteine ligands were known: alcohol dehydrogenase and aspartate transcarbamoylase. The identification of the transcription factor IIIA (TFIIIA) as a zinc protein (81) and designation of its repetitive metal-coordinating domains as *zinc fingers* (150) set the stage for a rapid pace of discoveries. Based on the proximity of zinc ligands and conservation of characteristic spacings between the ligands in primary sequences, it became feasible to search large data bases for the zinc finger and related motifs. Thus, assignment of zinc fingers was no longer made on the basis of an actual analysis of zinc but rather on the presence of a sequence motif. Present counts of zinc finger proteins in the human genome include 706 proteins with a classical zinc finger, 210 RING, 81 LIM, and 84 PHD domain proteins (140). This count does not even include the fact that some zinc finger proteins contain up to 36 fingers, nor does it include other enzymes or proteins with zinc/sulfur coordination, proteins such as MTs and related cluster motifs, interfacial binding sites, which are not recognizable from inspection of the sequence of the binding partners, or yet other uncharacterized zinc-binding domains (140). The zinc proteome is even larger, for it includes many sites that do not contain cysteine ligands. We are now witnessing another important change. Zn/S sites are not solely

static structures as one might have expected from their properties in isolation. The cell has harnessed the potential hidden in these structures for regulation using their reactivity, kinetics, and dynamics (85). Ligand-centered redox chemistry controls coordination dynamics and associated protein functions such as binding interactions. Sensitivity of Zn/S sites to redox species is employed in cellular redox signaling and makes these structures part of the cellular signaling network. The concept that redox signals are transduced opens another area for exploration, namely that of zinc signals as potent effectors of energy metabolism, mitochondrial function, signaling, and gene expression. Nature has solved the problem of zinc mobilization from tight binding sites by oxidative reactions on the cysteine ligands of zinc. Redox effects on zinc finger proteins provide an enormous potential for physiological regulation and interference with normal physiological function.

NOTE ADDED IN PROOF

Cys-11, His-37, Cys-41, and Cys-44 are ligands of zinc in the RsrA protein (section IIC), a member of the family of redox-sensing ZAS (Zn-containing anti-sigma factor) proteins. Cys-11 and Cys-44 form the trigger disulfide (178a).

A specific redox pair coupled to the T_R/T_O equilibrium (Fig. 11) has been identified. In a reaction coupled to thioredoxin, thionein can serve as a cofactor for methionine sulfoxide reductase (225a).

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ABBREVIATIONS

GSH, glutathione; GSSG, glutathione disulfide; IMS, intermembrane space; MT, metallothionein; MTF-1, metal response element (MRE)-binding transcription factor-1; NO, nitric oxide; Nrf2, NF-E2 related factor 2; PKC, protein kinase C; PTP: protein tyrosine phosphatase; T, thionein; T_O, oxidized thionein; T_R, reduced thionein.

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