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Metal and Redox Modulation of Cysteine Protein Function

Review

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In biological systems, the amino acid cysteine combines catalytic activity with an extensive redox chemistry and unique metal binding properties. The interdependency of these three aspects of the thiol group permits the redox regulation of proteins and metal binding, metal control of redox activity, and ligand control of metal-based enzyme catalysis. Cysteine proteins are therefore able to act as "redox switches," to sense concentrations of oxidative stressors and unbound zinc ions in the cytosol, to provide a "storage facility" for excess metal ions, to control the activity of metalloproteins, and to take part in important regulatory and signaling pathways. The diversity of cysteine's multiple roles in vivo is equally as fascinating as it is promising for future biochemical and pharmacological research.

Introduction

The amino acid cysteine endows proteins with an exceptional biochemistry due to the unique chemical characteristics of its thiol group [1, 2]. Its nucleophilicity, redox activity, and metal binding properties make cysteine an essential building block of many proteins and a key catalytic component of enzyme function. Considering redox activity and posttranslational modifications, cysteine residues with sulfur in numerous oxidation states have been identified in proteins (Figure 1). Among them, the thiol and disulfide oxidation states are probably best known, but modifications such as sulfenic, sulfinic, and sulfonic acids, disulfide-S-oxides, and a range of sulfur-centered radicals play an increasingly important role in biochemistry [2–4]. This variety of oxidation states is matched by the number of different redox mechanisms available to sulfur. For example, the thiol(ate) group can undergo nucleophilic attack, electron transfer, hydride transfer, hydrogen radical transfer, and oxygen atom transfer reactions [1, 5].

An additional property of cysteine is its unique metal binding ability. While the thiolate ligand strongly binds metal ions such as $Fe^{2+/3+}$, Zn^{2+}, Cd^{2+}, and Cu^{+} [6], oxidized forms of cysteine, such as sulfenic and sulfinic acids, have recently been implicated in the formation of iron and cobalt complexes in proteins [7]. Sulfur can accommodate a large number of bonds (through changes in oxidation state) and geometries resulting in very diverse structures with the same metal. For example, cysteine can function as a monodentate ligand to bind metals, as is found in the nitrogenase iron protein from *Azotobacter vinelandii*, which contains a [4Fe-4S] cluster where each iron is coordinated to a separate cysteine ligand [8]. Cysteine can bind more than one type of metal in a cluster, as exemplified by the recent characterization of carbon monoxide dehydrogenase from *Carboxydothermus hydrogenoformans*, which contains an active site [Ni-4Fe-5S] cluster where again each metal is ligated by a separate cysteine [9]. Cysteine can also act as a bidentate bridging ligand, as seen in the active site of the [NiFe] hydrogenase from *Desulfovibrio vulgaris* Miyazaki F, which contains four cysteine sulfur and four nonprotein sulfide (S^{2-}) ligands coordinated to the cluster. Two of the cysteines act as bridging ligands between the iron and nickel, and the remaining two cysteines coordinate the nickel [10]. Cysteine can also act as a bridging ligand between different subclusters. The iron-only hydrogenase from *Clostridium pasteurianum* contains an [4Fe-4S] subcluster coordinated by three cysteines and has a fourth coordinating cysteine ligand that bridges to the [2Fe] subcluster [11]. These versatile metal binding modes of the cysteine ligand are exemplified by the protein metallothionein, which contains two zinc/sulfur clusters. The α cluster (Zn_4Cys_{11}) binds four zinc ions with six terminal and five bridging cysteine ligands, whereas the β cluster (Zn_3Cys_9) binds three zinc ions with six terminal and three bridging cysteine ligands [12]. The resulting diversity in cysteine biochemistry gives rise to an extraordinary interplay of catalytic activity, redox behavior, and metal binding in proteins.

To approach this complex interaction in a systematic manner, this review starts with the chemistry of the thiol group, i.e., its nucleophilicity, redox activity, and metal binding properties. These three properties are then considered within the context of mammalian biochemistry. Rather than providing a comprehensive summary of cysteine biochemistry, the review focuses on situations where these three properties "coincide" in proteins, giving rise to redox control of cysteine's catalytic activity, redox control of metal binding, metal control of cysteine's catalytic activity, and cysteine control of a metal's catalytic activity.

Since these scenarios are illustrated by describing selected highlights, it should be mentioned from the outset that several of the examples discussed here are still under investigation and are, therefore, not entirely uncontroversial. In addition, some equally interesting aspects of cysteine biochemistry (e.g., thiol:disulfide oxidoreductases, iron/sulfur proteins [13]) cannot be ad-

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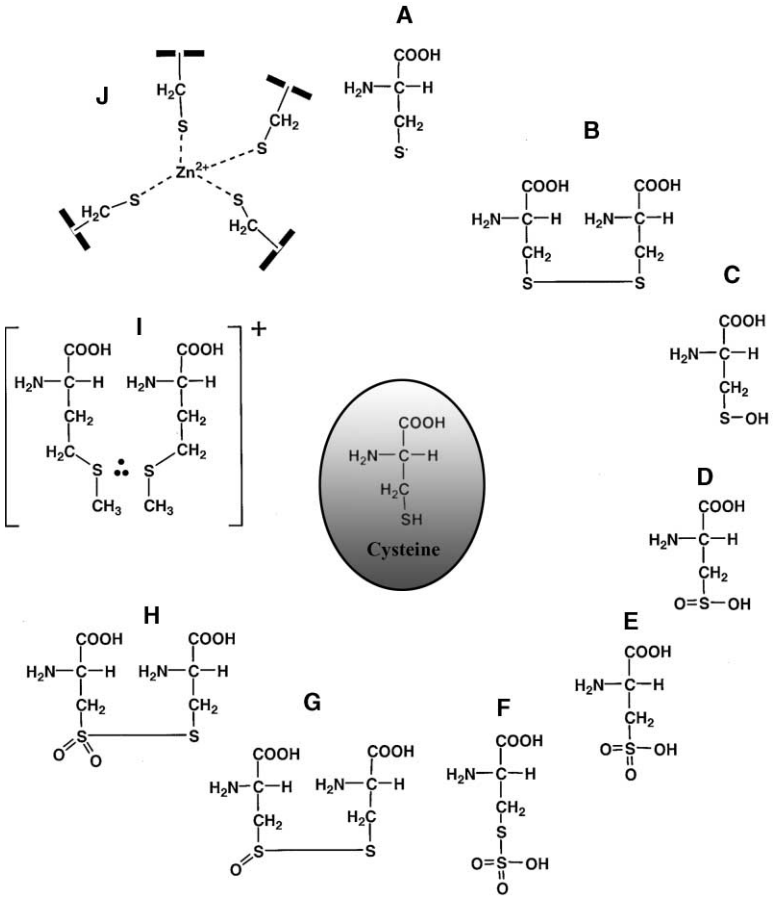

Figure 1. Important Posttranslational Cysteine and Methionine Modifications Found in Peptides and Proteins

Formal oxidation states are shown in brackets.

(a) Cysteinyl radical (−1), (b) cystine (i.e., "cysteine disulfide") (−1), (c) cysteine sulfenic acid (0), (d) cysteine sulfinic acid (+2), (e) cysteine sulfonic acid (+4), (f) cysteine-S-sulfate (+5), (g) cystine-S-monoxide (+1), (h) cystine-S-dioxide (+3), (i) methionine disulfide radical cation with a three electron bond (−3/2), and (j) a tetrahedral $ZnCys_4$ complex (−2). Further details are given in the text. For a more detailed list of cysteine modifications, see [5].

dressed here, and literature references of recent reviews in these areas are provided throughout the text.

The first section of this review (Redox Control) looks at examples of how catalytic activity is controlled by the redox state of active site cysteine residues. Changes in the sulfur oxidation state of cysteine influences the activity of many proteins [2, 14]. The details of selected examples discussed in the text are summarized in Table 1. It also has dramatic effects on the amino acid's metal binding properties, and these are discussed in the second section (Ligand Oxidation and Metal Release). While the first two sections focus on the redox control of metal binding and differences between ligand-controlled metal activity and metal-controlled ligand activity in proteins, the interaction of cysteine and metals is also important from the metal's point of view. Zinc/sulfur complexes play a prominent role in the maintenance of zinc homeostasis inside the cell, an emerging regulatory mechanism that is discussed in the third section (Regulation of Zinc Homeostasis).

A deeper understanding of these control mechanisms has also stimulated the exploration of new avenues in pharmacological research. The fifth section (Drug Design) briefly considers recent advances in the design of drug prototypes that either mimic or interfere with the processes discussed in the sections Redox Control, Ligand Oxidation and Metal Release, and Regulation of Zinc Homeostasis. The Summary provides a brief recap and outlook on future developments in this emerging area of biochemical research.

Redox Control of Cysteine's Catalytic Activity

A number of cysteine-containing proteins such as the thioredoxins (Trx) and enzymes such as glutathione reductase (GR) require redox-active cysteine residues for their activity [15–18]. In contrast, a change in the oxidation state of sulfur must be avoided in redox-sensitive cysteine proteins in which the reduced state of the thiol is an absolute requirement for activity. Oxidative enzyme inhibition is frequently observed in proteins under conditions of oxidative stress, where reversible disulfide formation and irreversible cysteine oxidation to sulfinic acids has been observed [19, 20].

More recently, the oxidative modification of cysteine residues in proteins has been implicated in cellular signaling and regulatory pathways [20]. This notion is highly important, since it assigns a specific biochemical role to oxidative, posttranslational cysteine modification (e.g., during oxidative stress [21]) that goes beyond the apparently "random" cysteine oxidation frequently observed under aerobic conditions in vitro. The significance of controlled cysteine oxidation is further underlined by the increasing number of cysteine enzymes that redox cycle between the thiol and other, "unusual," sulfur oxidation states such as the thiyl radical and sulfenic acid. It is therefore helpful to briefly consider the most common

Table 1. Control of Cysteine Protein Activity

Protein	Role of Cysteine Residue	Redox States of Sulfur	Role of Metal Ions	Modulation of Activity
ADH	1 catalytic and 1 structural zinc/sulfur complex	thiol	catalytic (Zn^{2+})	cysteine oxidation (↓) Zn^{2+} removal (↓)
Agro-bacterial esterase	essential disulfide	disulfide	unknown	disulfide formation (↑)
CPA	none	N/A	1 catalytic, 1 inhibitory Zn^{2+} site	D-cysteine (↓) Zn^{2+} removal from inhibitory (↑) or catalytic site (↓) Cd^{2+} exchange at catalytic site (↓)
Caspase-3	catalytic	thiol	inhibitory (Zn^{2+})	Zn^{2+} (↓) chelator (↑) cysteine oxidation (↓)
GAPDH	catalytic	thiol	inhibitory (Zn^{2+})	Zn^{2+} (↓) chelator (↑) cysteine oxidation (↓)
MMP	inhibitory	thiol	catalytic (Zn^{2+})	cysteine (pro-MMP) (↓) cysteine oxidation (↑) metal ions (?) TIMP binding (↓) Zn^{2+} chelators (↓)
MT	zinc/sulfur complexes	thiol	storage (Zn^{2+}) oxidative stress sensor (?)	oxidative metal release metal exchange (Cd^{2+})
MTF-1	CCHH zinc finger	thiol	Zn^{2+} sensor	oxidative Zn^{2+} release (?)
Nox	catalytic	thiol/sulfenic acid	unknown	unknown
NHase	ligand (Fe^{3+},Co^{3+})	sulfenic, sulfinic acid	catalytic	sulfenic acid modifiers (↓)
PTP	catalytic	thiol	inhibitory (Zn^{2+})	Zn^{2+} (↓) chelator (↑) cysteine oxidation (↓)
Sp1	CCHH zinc finger	thiol	structural (Zn^{2+})	oxidation (↓) Zn^{2+} chelator (↓)

A selection of cysteine proteins whose activity is significantly modulated by redox processes, adventitious metal binding, chelators, and thiols. Details are found in the text.

oxidation pathways and redox modifications of cysteine found in proteins.

Cysteine Oxidation in Proteins: Pathways and Species

Numerous oxidized cysteine species have been found in vivo and in vitro, among them thiyl radicals, disulfides, persulfides, sulfenic acids, sulfinic acids, sulfonic acids, polysulfides, selenosulfides, disulfide-S-monoxides, and disulfide-S-dioxides (Figure 1). The formation and (bio)-chemical properties of these thiol modifications provide an extensive field of biochemical research on its own that has been recently reviewed [4, 22]. In short, different oxidative modifications of cysteine residues in proteins occur as a result of the thiol(ate) group's ability to transfer electrons and atoms, act as a nucleophile, and undergo radical reactions. The following examples illustrate the variety of redox mechanisms and resulting oxidation states found for cysteine in vivo.

The low redox potential of cysteine (ranging from around −270 mV to −125 mV versus NHE in most proteins [23, 24]) allows rapid *electron transfer* from cysteine, resulting in thiyl radical and disulfide formation [25]. The reverse reaction is frequently observed in proteins such as human glutathione disulfide reductase (GR) and thioredoxin reductase (TrxR), where a two-electron transfer from $FADH_2$ reduces a cystine disulfide bond to two cysteine thiols [18, 26]. Chemically, electron transfer to a disulfide is a reversible reaction that can be studied electrochemically [27–29], while other redox mechanisms of sulfur are considerably more difficult to investigate. From a mechanistic point of view, it is interesting to note that the oxidation of two thiols to a disulfide can proceed via two one-electron transfers with subsequent dimerization, while the reduction of the disulfide proceeds via one two-electron transfer.

In many proteins, cystine reduction involves electron transfer while cystine formation does not. This apparent paradox is the result of cysteine's ability to participate in different redox mechanisms. In GR, electron transfer reduces an active site disulfide that is formed via a thiol/disulfide exchange reaction with glutathione disulfide (GSSG). This process consumes NADPH and produces reduced glutathione (GSH). GR therefore connects the cell's energy metabolism (NADPH) with the maintenance of its redox balance, determined by the GSH:GSSG ratio (usually in the range of 100:1) [30, 31].

Electron transfer from cysteine is observed between its thiol group and metal ions such as Cu^{2+} and Fe^{3+}. Cysteine residues in the zinc/sulfur protein metallothionein (MT), for example, are directly oxidized by cytochrome *c* [32], while other cysteine oxidation pathways in the presence of metal ions involve radical species. In the absence of normal substrate, redox-active metal ions in metalloenzymes can undergo electron transfer reactions with oxygen, resulting in the generation of superoxide and hydrogen peroxide. In addition, hydroxyl radicals can be formed via Fenton-type chemistry from hydrogen peroxide and metal ions [33].

Cysteine residues are particularly susceptible to these oxidative stressors, forming a variety of posttranslational modifications. The one-electron oxidation of cysteine by Fe^{3+} or Cu^{2+} ions and radical species such as the hydroxyl radical, superoxide, and nitric oxide initially results in (thiyl, $RS^{\bullet}$) radical formation. The latter can combine with a second radical to form nonradical species such as disulfides (RSSR), sulfenic acids (RSOH), sulfinic acids (RS(O)OH), or, in the case of NO, an S-nitrosylated cysteine (RSNO) (Figure 1).

This complex interplay between cysteine and redox-active metal ions is exemplified in the case of phenylalanine-sensitive 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (DAHPS) from *Escherichia coli*. This enzyme requires a Cu^{2+} cofactor and is unstable in the absence of its substrate [34]. The auto-inhibition process is ascribed to the oxidation of two cysteine residues (Cys61 and Cys328) at the active site to cysteinyl radicals. The reaction is initiated by one-electron transfer from cysteine to Cu^{2+}, forming Cu^{+} and the first cysteinyl radical. A hydroxyl radical is produced by the reaction of Cu^{+} and H_2O_2, regenerating Cu^{2+}. The hydroxyl radical then reacts with the second cysteine residue, forming another cysteinyl radical and water. The resulting two cysteinyl radicals dimerize to form a disulfide. This is confirmed by incubation with DTT, which restores enzymatic activity [34].

The radical biochemistry of cysteine itself is becoming increasingly important [3]. *Cysteine-based radicals* can be formed by long-range one-electron transfer reactions and short-range hydrogen atom abstraction and include species such as thiyl, sulfinyl, and sulfonyl radicals, disulfide radical anions, and a range of peroxyl radicals, many of which are likely to exist during oxidative stress and in the presence of redox-active metal ions [4, 35]. Sulfur-centered radicals are also increasingly found as part of enzymes' catalytic cycles (e.g., RNRase superfamily, *E. coli*, pyruvate formate lyase [PFL], and *Thauera aromatica* benzylsuccinate synthase [BSS] [36–38]), where they provide an exciting new area of biochemical radical research.

In contrast, peroxidation of thiols leads to the formation of sulfenic and sulfinic acid species whose properties are very different from the disulfide state. Such *oxygen atom transfer reactions* are observed in redox enzymes such as *Streptococcus faecalis* NADH peroxidase (Npx), NADH oxidase (Nox), and human peroxiredoxins (Prx) [39, 40], which function via a cysteine (Cys-SH)/cysteine sulfenic acid (Cys-SOH) redox couple. The catalytic cysteine residues of these enzymes are oxidized by hydrogen peroxide to cysteine sulfenic acid (in the case of Nox, also involving the formation of peroxyflavin). The reduction of the sulfenic acid can proceed via two distinct mechanisms both found in vivo: via an exchange reaction involving two thiol equivalents (e.g., Prx) or via hydride transfer from $FADH_2$ (e.g., Npx and Nox) [39].

Most intracellular disulfides are, however, formed and broken by a mechanism not involving any electron or oxygen atom transfer. *Thiol/disulfide exchange reactions* are *nucleophilic substitution reactions* of thiolate RS^{-} on the disulfide R'SSR', resulting in a mixed disulfide RSSR' (with a formally oxidized RS thiol) and reduced $R'S^{-}$. These reactions are widespread in vivo (e.g., found in thiol:disulfide oxidoreductase enzymes [15]) and also contribute to very important posttranslational protein modification processes.

The formation of mixed disulfides between protein thiols and low molecular mass thiols such as cysteine and GSH (S-glutathionylation) is thought to be part of cellular signaling [19]. Changes in the cellular redox balance trigger signal transduction pathways by modifying the oxidation state of cysteine residues in participating proteins, shuttling between cysteine, cysteine disulfides, cysteine sulfenic, and cysteine sulfinic acids. Redox modifications can exert a variety of effects on proteins ranging from inactivation of a catalytic residue (e.g., mammalian protein tyrosine phosphatase [PTP]) to extensive structural changes (e.g., *Escherichia coli* OxyR, Figure 2) [19, 41].

In addition, S-thiolation reactions have recently been considered as a temporary protective mechanism utilized by enzymes during oxidative stress that prevents irreversible modification of their critical active site thiol [2]. Mammalian Protein kinase C, creatine kinase, glucocorticoid receptors, and DNA binding by transcription factors such as c-Jun are a few examples of functionally very diverse proteins, all of whose activity are affected by protein S-thiolation/glutathionylation [42–45].

Like S-glutathionylation, other nucleophilic substitution reactions also result in reversibly modified cysteine residues that might play a role in enzyme regulation. Proteins such as mammalian hemoglobin, albumin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), caspase, PTPs, and alcohol dehydrogenase (ADH) can be oxidatively modified by S-nitrosylation to form R-SNO species [46–50]. This reaction alters the activity of these proteins, suggesting that this is a possible method of protein regulation in vivo—in line with the known function of NO as a cellular signaling molecule. The presence of large amounts of GSH in cells implies that nitrosoglutathione (GSNO) is another important form of nitric oxide in vivo. GSNO can be nucleophilically attacked by proteins' cysteine residues at its sulfur or nitrogen atom, resulting either in protein S-nitrosylation or S-glutathionylation as observed for phosphorylase b, H-ras, and carbonic anhydrase III [51].

Although many cysteine modifications irreversibly abolish protein function, the reversible (e.g., S-thiolation, sulfenic acid formation) and some of the irreversible (e.g., sulfinic and sulfonic acid formation) processes are increasingly considered as regulatory processes rather than just random inhibition of protein activity [2, 20, 39]. This regulatory role is becoming important during

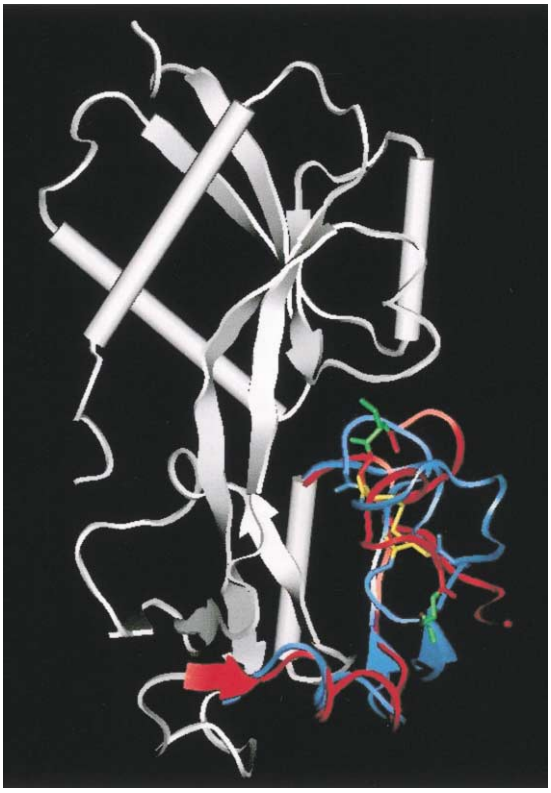

Figure 2. A Diagrammatic Representation of the Crystal Structure of OxyR

Structural changes of the "redox switch" OxyR upon oxidation (reduced and oxidized forms of OxyR, PDB IDs 1I6A and 1I69, respectively) [175]. The mechanism of activation of OxyR involves the hydrogen peroxide-induced formation of a disulfide between Cys199 and Cys208. The bulk of the structure (white) is unaffected by this activation mechanism. In the reduced form (red; Cys199 mutated to Ser), these two residues (green) are 17 Å apart, and in the oxidized form (blue), formation of the disulfide (yellow) results in major structural rearrangement that includes formation of a new β strand. The figure was produced using QUANTA2000 [176]. α helices are shown as cylinders, β sheets as arrows, and the cysteine and cystine residues are shown as liquorice.

normal cell function, apoptosis, aging processes, and pathological conditions such as oxidative stress [2, 39, 52–54]. Among the numerous redox-sensitive cysteine-containing proteins, cysteine peptidases and dehydrogenases best exemplify oxidative and redox control. These enzymes will therefore be discussed in more detail.

Redox Control of Cysteine Proteases and Dehydrogenases

Many enzymes utilize a catalytic cysteine residue in the form of a thiolate anion as a nucleophile in reactions ranging from thiol exchange redox reactions (e.g., Trx, GR) to proteolytic reactions as found in cysteine proteases. The family of cysteine proteases consists of a range of different enzymes with different overall tertiary structures and distinct biological functions. These include the human apoptosis protease caspase-3, cathepsin F (implicated in processing of the invariant chain associated with Major Histocompatability Complex [55]), cathepsin B (implicated in the remodeling of the extracellular matrix during tumor cell invasion [56]), and the calpains (implicated in cytoskeletal remodeling processes, cell differentiation, apoptosis, and signal transduction) [57].

The catalytic mechanism of cysteine proteases based on papain has been reviewed by Brocklehurst et al. [58]. It is similar to that of the serine proteases, where cysteine acts as a nucleophile and a histidine residue acts as a proton donor (Figure 3A for general mechanism). Nucleophilic attack by cysteine results in formation of the first tetrahedral species followed by formation of an acyl enzyme intermediate and release of the amine product. Attack by a water molecule results in formation of the second tetrahedral species, which is followed by polypeptide chain release, leaving the enzyme back in its original state to carry out another round of catalysis. This mechanism is based on a combination of covalent and acid-base catalysis. In addition to the cysteine-histidine catalytic "diad," some proteases contain an acidic residue, usually aspartic acid or asparagine [59]. This residue is suggested to stabilize and orient the imidazolium ring and favor the movement of positive charge from the cysteine to histidine residue of the active site.

The active site cysteine residue can be readily oxidized as observed from the additional electron density associated with this residue in several high-resolution crystal structures such as archaeal GAPDH and pyrrolidone carboxyl peptidase (PCP) [60, 61]. The in vivo implications of this oxidative inhibition of protease activity are particularly important in the case of the caspase enzymes, cysteine proteases located in the cytoplasm, and mitochondria, which play a critical role in the apoptotic pathway. Human caspase-3 is directly involved in the process of programmed cell death, where it inactivates proteins involved in cell repair and homeostasis. The crystal structure for this protease is now available [62]. The mature form of the enzyme is a complex of two polypeptide chains: p12 is involved in substrate binding and p17 provides the catalytic residues. Oxidative changes to caspase activity are primarily mediated by NO and are also dependent on the presence of free iron and the intracellular redox potential [63]. Mitochondrial caspases-3 and -9 are frequently S-nitrosylated at the active site cysteine, a process that inhibits proteolytic activity and their ability to induce apoptosis and inflammation [64]. The majority of cytoplasmic enzymes, however, are not nitrosylated, which suggests that movement of these caspases into the cytoplasm does not favor stability of S-NO, possibly a method of reversible caspase activation by denitrosylation [65]. This underlines the notion that oxidative cysteine modification is not a random process resulting in protein inhibition, but rather a process suitable to control enzymatic activity within the redox environment of the cell.

In contrast to this kind of reductive activation, other hydrolytic enzymes appear to be activated by the oxidation of cysteine residues leading to the formation of disulfide bonds essential for enzyme activity [66]. Disulfide formation is considered to be a common mechanism of protein stabilization and folding, and primarily proteins exported from the cell are rich in disulfide bonds. These are formed at the correct positions in the folded

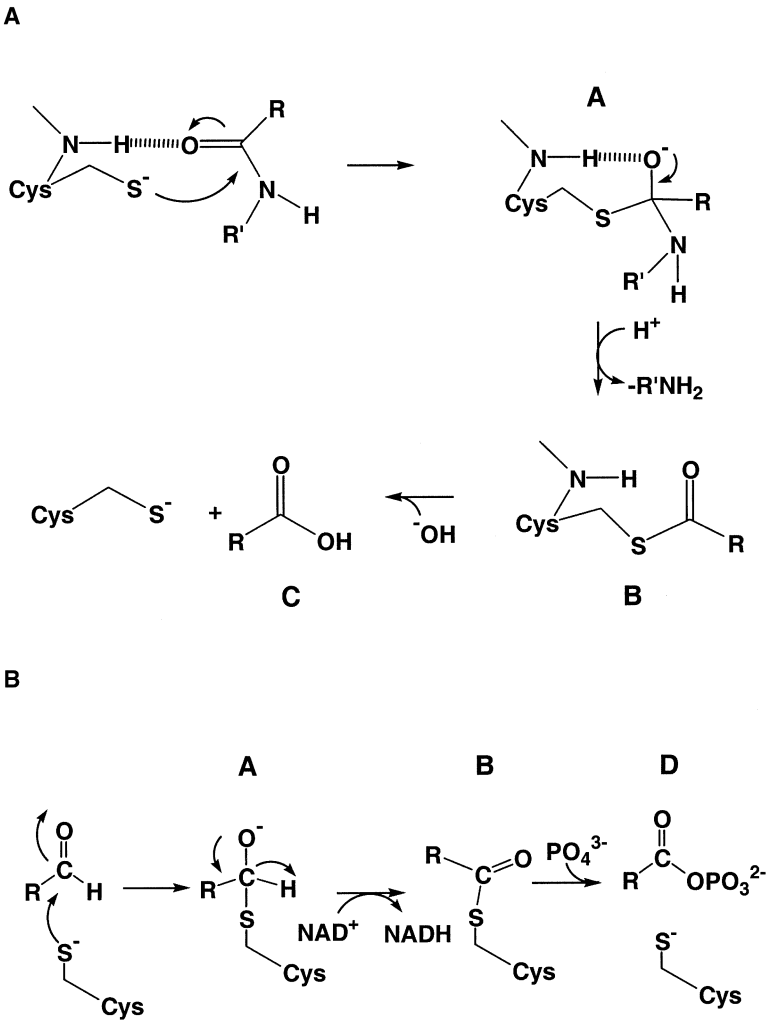

Figure 3. Catalytic Cycles of Cysteine Proteases and Dehydrogenases

Panel (A) shows cysteine proteases, and panel (B) shows dehydrogenases. Initial attack of the cysteine thiolate anion results in the formation of a tetrahedral species (A). Collapse of the tetrahedral species results in the formation of the acyl-enzyme (thiolester) intermediate (B). Nucleophilic attack by either the phosphate or hydroxyl anion results in the formation of the carboxylic acid (C) or phosphoester (D) and regeneration of the thiolate anion [58, 177].

protein by enzymes such as the thiol:disulfide oxidoreductase enzyme protein disulfide isomerase (PDI) found in the endoplasmic reticulum of eukaryotic cells [67].

Oxidative activation by disulfide bond formation is, however, a novel mechanism that has been recently proposed to occur in an agrobacterial esterase enzyme that has been studied crystallographically at atomic resolution [66]. A disulfide bond between two adjacent residues needs to be formed to complete the "oxyanion hole" for binding of the tetrahedral intermediate in the reaction mechanism. It is possible that human enzymes are also regulated in this way, and kinetic studies combined with high-resolution enzyme structures will explore this issue further.

In a manner similar to cysteine proteases, several dehydrogenase enzymes are affected by the redox state of an active site cysteine residue. Mammalian muscle GAPDH catalyzes the oxidation and subsequent phosphorylation of aldehydes to acyl-phosphates while reducing NAD^+ to NADH. Its catalytic cycle involves a crucial hydride transfer step that requires an activated aldehyde substrate. The enzyme's active site contains a cysteine that attacks the aldehyde to form a highly reactive tetravalent thioether intermediate (Figure 3B). A hydride is transferred to NAD^+ to form a thioester at the active site. The latter is cleaved by nucleophilic attack of phosphate, resulting in the formation of a phosphoester with regeneration of the active site cysteine. Related enzymes, such as yeast aldehyde dehydrogenase (ALDH), follow a similar redox mechanism, and these enzymes are inhibited by oxidizing agents that modify the catalytically active cysteine residue. Among those biologically relevant oxidizing species, peroxynitrite [68] and "reactive sulfur species" [4, 69] have recently been investigated in the context of dehydrogenase regulation during oxidative stress.

While the effects of oxidation on the activity of these proteins are well understood, the formation of "unusual" oxidation states in the sheltered environment of the active site has led to a few surprises. A stable sulfenic acid has been observed in the active site of papain [70, 71] as well as other cysteine proteases [72] and GAPDH [73, 74]. Cysteine sulfenic acids also form part of catalytic redox cycles in enzymes. Crystallographic data from the native oxidized crystal structures of *Streptococcus faecalis* NADH oxidase and NADH peroxidase and human peroxiredoxins have shown the formation of a cysteine sulfenic acid at the active site [19, 39, 70, 75].

The active sites of these enzymes are therefore able

to sustain the formation of the highly unstable cysteine sulfenic acid, a thiol modification that is notoriously difficult to achieve in chemical synthesis [76–78]. In contrast, overoxidation of the sulfenic acid to the more stable sulfinic acid provides an additional sulfur acid modification that is, for example, found in bacterial nitrile hydratase (NHase) from *Rhodococcus sp N-771* [7].

While caspase-3 and GAPDH activity are directly affected by the modification of a catalytic cysteine, zinc/sulfur enzymes frequently lose their catalytic activity by oxidative release of the catalytic metal ion. Cysteine oxidation is not always, however, detrimental to enzyme activity, as the next section illustrates.

Ligand Oxidation and Metal Release in Metalloproteins

Several metalloproteases and mammalian liver alcohol dehydrogenase (ADH) contain an active site zinc/sulfur complex that is under redox control. There are two possible regulatory scenarios. First, the role of the thiolate ligand is to bind a zinc ion essential for catalytic activity. Oxidation of the ligand would result in loss of activity due to metal release from the active site. Second, the ligand occupies a coordination site on the metal that is essential for substrate binding. On oxidation of this ligand, the binding site for substrate becomes available, leading to activation of the enzyme (on condition that the other ligands, such as histidine, are not affected by oxidation). The oxidative disintegration of metal/sulfur complexes can, therefore, be seen from two different perspectives: enzyme inhibition by release of an essential metal ion and enzyme activation by release of a (superfluous) ligand. The first scenario is exemplified by ADH and metallo-β-lactamases, while the second plays a major role in the regulation of the activity of matrix metalloproteases (MMPs).

Zinc Release

Mammalian ADH catalyzes the oxidation of ethanol to acetaldehyde under alkaline conditions. The most studied is horse liver ADH, which is an 80 kDa dimeric enzyme with two tightly bound zinc atoms (one catalytic and one structural) and one NADH binding site per subunit. The structural zinc is bound by Cys97, Cys100, Cys103, and Cys111 in a tetrahedral geometry, while the catalytic zinc ion is ligated by Cys46, Cys174, His67, and a water molecule with an additional bond to Ser48. As a ligand, the water molecule is easily exchangeable for substrate. Crystallography has shown that in the enzyme-NAD^+-alcohol ternary complex [79], the OH group of the alcohol binds to the zinc through an inner coordination sphere, with the side chains of Ser48 and Phe93 effectively locking the alcohol into position for hydride transfer from the alcohol to the NAD^+ [80].

Not surprisingly, the high number of cysteine ligands makes the zinc binding sites in ADH sensitive toward oxidation that causes the release of zinc and subsequently enzyme inhibition [69, 81]. Like GAPDH, ADH is readily inhibited by NO, and nitrosothiolation at the catalytic site is accompanied by zinc release [48]. From a pharmacological point of view, oxidation and subsequent inhibition of liver ADH can be used to sensitize the organism against ethanol. As a consequence, thiol-specific oxidizing agents, such as disulfiram (tetraethylthiuram disulfide, antabuse), have been used in the past to treat alcoholism by inhibiting ADH (and other enzymes) in patients [82, 83].

It should be noted, however, that neither cysteine oxidation nor metal removal from active sites is always detrimental to enzyme activity, as the example of the agrobacterial esterase enzyme has already illustrated. The next section discusses examples of enzyme activation by removal of unwanted metals from the active site.

Ligand Release

In contrast to these hydrolases, the matrix metalloprotease (MMP) family of proteases is activated by cysteine oxidation. For normal growth and development, cells must interact with the extracellular matrix (ECM) that is continually modified by proteolytic systems. The latter alter the constitution of the ECM structure and therefore regulate cell growth, differentiation, and cell death. Uncontrolled degradation leads to the development of pathological conditions including inflammation, arthritis, and cancer [84]. Human MMPs are zinc-dependent endopeptidases able to cleave a variety of ECM components. They are synthesized in an inactive form inside the cell and exported to the ECM, where they are activated by proteolytic cleavage of the propeptide region. The molecular weight ranges from 62 to 130 kDa between various species and proteolyzed forms of the enzyme. Recent studies have shown that the activity of these enzymes can also be regulated by thiol compounds and redox reactions [85–87].

MMPs are organized into three major domains based on structure: a catalytic domain, a carboxy-terminal hemopexin-like domain, and a propeptide region. The catalytic domain of MMP enzymes contains one catalytic and one structural zinc ion and at least one calcium ion [84]. The catalytic zinc ion is bound by three highly conserved histidine residues. It has a vacant coordination site that is occupied in the active enzyme by a highly polarized water molecule that acts as the nucleophile during the catalytic cycle of the enzyme. In the inactive form, however, this coordination site is occupied by a thiolate ligand from the propeptide region (Figure 4A). The latter is 80–90 amino acids in length and contains a redox-sensitive cysteine residue within the highly conserved PR**C**GXPD sequence, which interacts with the catalytic zinc, hence functioning as a "stopper" of activity [84].

The biological role and mechanism of oxidative MMP activation is still under investigation. It was initially found that pro-MMP-1, -8, and -9 were activated by peroxynitrite in the presence of GSH, indicating that the S-glutathionylation of the propeptide region (via the formation of glutathione disulfide-S-monoxide) was taking place [87]. More recently, it has been found that pro-MMP-9 is activated by S-nitrosylation and then induces neuronal apoptosis in vitro [85]. In related in vivo experiments, MMP-9 colocalized with neuronal nitric oxide synthase during cerebral ischemia. Mass spectrometry identified the active derivative of MMP-9, both in vitro and in vivo, which was found to contain a stable sulfinic or sulfonic acid, whose formation was initiated by S-nitrosylation [85].

Similarly, the activation of pro-MMP-7 in the artery wall can be controlled by myeloperoxidase and the production of hypochlorous acid [86]. Mass spectrometric

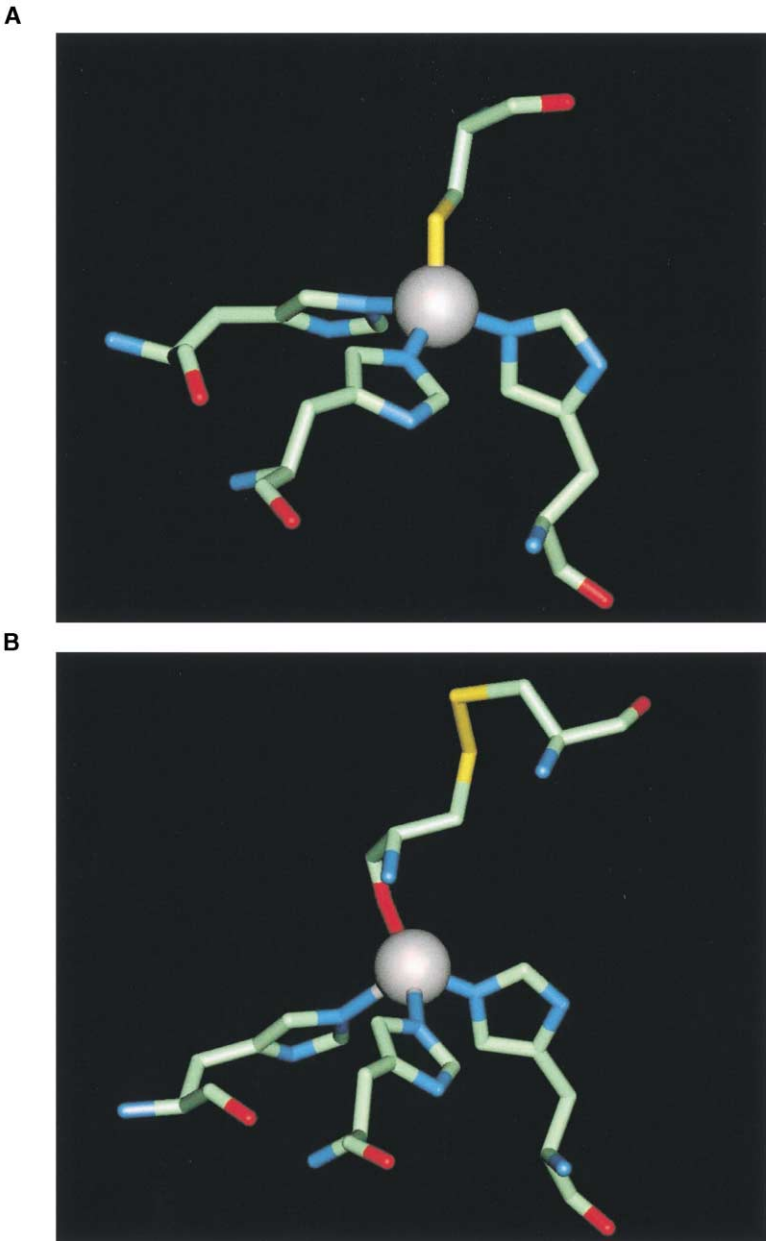

Figure 4. Ligand Control of MMP Activity

Activity of these zinc enzymes can be modulated by ligand binding in two different ways. Part (A) shows inhibition of pro-MMP2 by coordination of a cysteine ligand of the prodomain to catalytic zinc (PDB ID 1GXD) [178]. The formation of a metal-ligand bond is not, however, a requirement, and part (B) indicates how the formation of the Membrane-type MMP1/TIMP complex results in blockage of substrate access to the active site (PDB ID 1BUV) [179]. Figure produced using QUANTA2000 [176].

studies of human atherosclerotic lesions (both in vitro and in vivo) have shown that the thiol residue of the cysteine in the enzyme's prodomain was converted to a sulfinic acid. As such, thiol oxidation was associated with autolytic cleavage of pro-MMP-7, suggesting that oxidation activates the latent enzyme [86].

Like the MMP enzymes, the human zinc protease carboxypeptidase A is also inhibited by "undesired ligands" such as D-cysteine, which replaces the hydroxyl ion at the active site [88]. Oxidative removal of thiols from the active site of this enzyme has, to the best of our knowledge, not yet been studied. Neither has thiol ligand removal by excess zinc ions, a possible theoretical alternative to ligand oxidation.

MMPs are also inhibited by a range of proteins known as "tissue inhibitors of metalloproteases" (TIMPs) [89]. Interestingly, TIMPs are peptides of approximately 20 kDa that contain several conserved cysteine residues (12 in the case of TIMP-1 and TIMP-2), all of which are in the disulfide state. These disulfides are essential for the structural integrity of the TIMP proteins and their inhibitory activity. The crystal structure of the complex between human stromelysin-1 (MMP-3) and human TIMP-1 shows that the amino group of the TIMP cysteine residue (Cys-1) blocks the active site zinc of MMP-3, inhibiting its activity by exclusion of water [89]. A similar interaction is observed for the cysteine residue (Cys-1) of TIMP-2 binding to membrane-type-1 MMP (Figure 4B). Paradoxically, while oxidation of the inhibitory thiolate ligand in pro-MMPs activates the enzyme, reduction of the disulfide bonds in TIMP would, at least hypothetically, also lead to activation via changes in TIMP structure and the resulting dissociation of the MMP-TIMP complex.

It should be mentioned that, while oxidative MMP activation provides certain similarities with the oxidative activation of the agrobacterial esterase enzyme mentioned above, the activation pathways are completely different. MMP activation involves ligand removal from a catalytic metal, while the esterase is activated by the formation of an essential disulfide bond. The following section looks at yet another activation mechanism based on the dismantling of an "unwanted" metal/zinc complex—this time with the metal (and not the ligand) as inhibitor.

Metal Poisoning

Hydrolases and dehydrogenase enzymes with active site cysteines provide an interesting paradox: while some of these enzymes require zinc ions for their activity (e.g., metallo-lactamases, ADH), others are inhibited by zinc (e.g., caspase-3, GAPDH) [90–92]. Zinc inhibition is observed when metal ions bind directly to cysteine at the active site, and this unwanted metal binding is of particular interest since it is frequently an unwelcome result of cysteine chemistry. While the thiolate group in enzymes such as GAPDH and caspase-3 catalytically acts as a nucleophile, it is by definition also an excellent ligand for zinc ions. "Random" zinc binding to cysteine residues in these proteins is therefore frequently observed in vitro and, as the inhibitory effects of zinc ions on fungal growth show, might play a role in vivo [93]. Other divalent metals such as copper and cadmium have also been shown to inhibit GAPDH from *Xenopus laevis* and *Pleurodeles waltl* by binding to the active site cysteine [94].

A second mechanism of metal poisoning is the substitution of the catalytic metal by an inhibitory metal, leading to loss of enzyme activity. In human porphobilinogen synthase, for example, Pb^{2+} displaces the active site Zn^{2+}, which is ligated by cysteine. Pb^{2+} disfavors substrate binding, which in turn leads to loss of activity [95].

It should be mentioned that a third mechanism for protein inhibition by metal ions is based on genuine inhibitory sites in enzymes. These sites are different from the active site and do not necessarily involve cysteine residues. Carboxypeptidase A, for example, has an inhibitory zinc binding site, and occupation of this site by zinc ions reduces enzyme activity [96–98]. A fourth mechanism involves cysteine oxidation by redox-active metal ions and has been discussed in the Redox Control section.

The precise role of metal poisoning in vivo is unclear. Metal binding to catalytic cysteine residues is reversible and can be reversed in the presence of chelating agents, such as thionein for zinc [92]. This kind of metal binding might be understood as part of a control mechanism in the presence of elevated concentrations of unbound metal ions. Alternatively, it might provide a protective mechanism for active site cysteines: under normal conditions, concentrations of free zinc in the cytosol are usually too low to cause widespread cysteine enzyme inhibition. In the presence of oxidative stress, however, zinc is released from proteins such as metallothionein (MT) [99], and this increase in cytosolic zinc levels might then be sufficient to allow the formation of zinc/sulfur bonds at the active sites of enzymes such as GAPDH and caspase-3 [92]. Interestingly, this might prevent rapid cysteine oxidation and subsequent irreversible enzyme inhibition that would otherwise occur under these conditions.

The interaction of cysteine proteins with free metal ions clearly warrants further investigations. It also emphasizes the importance of a tightly controlled metal homeostasis inside the cell.

Regulation of Zinc Homeostasis

Oxidative disintegration of metal complexes has already been mentioned as an inhibitory mechanism for ADH and as part of a possible activation mechanism for MMP proteins. Oxidative regulation of metal binding is particularly important in the control of the level of available metal ions inside the cell. Metal trafficking and the intracellular control of metal homeostasis have become increasingly important topics in biochemistry during the last decade [100] and the regulatory system for maintaining the intracellular zinc homeostasis is slowly becoming apparent. This section, therefore, discusses the emerging role of zinc/sulfur bonds in the sensing and storage of zinc, with focus on the role of metallothionein in maintaining the cellular zinc homeostasis.

Thionein, Metallothionein, and the Intracellular Zinc Homeostasis

Metallothionein is a major zinc binding protein that is thought to play a key role in maintaining the intracellular zinc homeostasis. An important property of the MT zinc store is that it can provide zinc where needed and scavenge heavy metals such as cadmium and mercury [101]. Although discovered more than 45 years ago [102], the precise biological role of MT has long remained illusive [103]. It has now been shown that the expression of MT's apo-form, thionein, is controlled by a feedback mechanism for the control of free zinc ions in the cytosol. This mechanism involves sensing the concentration of unbound ("free") zinc by a zinc finger protein, expression of thionein, zinc storage in MT, zinc transfer, and, possibly, oxidative zinc release (Figure 5) [104].

It should be mentioned from the outset that this regulatory mechanism is still under investigation and the precise function(s) of MT in vivo are still a matter of significant controversy [104]. This also includes the link of MT with the two families of zinc transporters that have been identified in eukaryotes, the Cation Diffusion Facilitator (CDF) family and the ZIP family. The CDF family (members of which include ZnT-1 to ZnT-7) mediates zinc movement out of cells or into intracellular compartments for storage and their role has recently been reviewed elsewhere [105–109]. Members of the ZIP transporter family are found in most organisms. They transport zinc ions into mammalian cells and have also been reviewed [110, 111].

Sensing the Concentration of Free Zinc Ions in the Cytosol. The human zinc finger protein metal response element binding transcription factor 1 (MTF-1) is an 80 kDa transcription factor containing six Cys_2His_2 (CCHH) zinc fingers [112]. Upon binding zinc, MTF-1 translocates to the cell's nucleus, where it binds to the metal responsive element (MRE) of DNA with an apparent DNA binding constant of $K_{app} \approx 3.8 \times 10^8\ M^{-1}$ (pH 7.0) [113]. Zinc binding to MTF-1 is reversible, with activation of

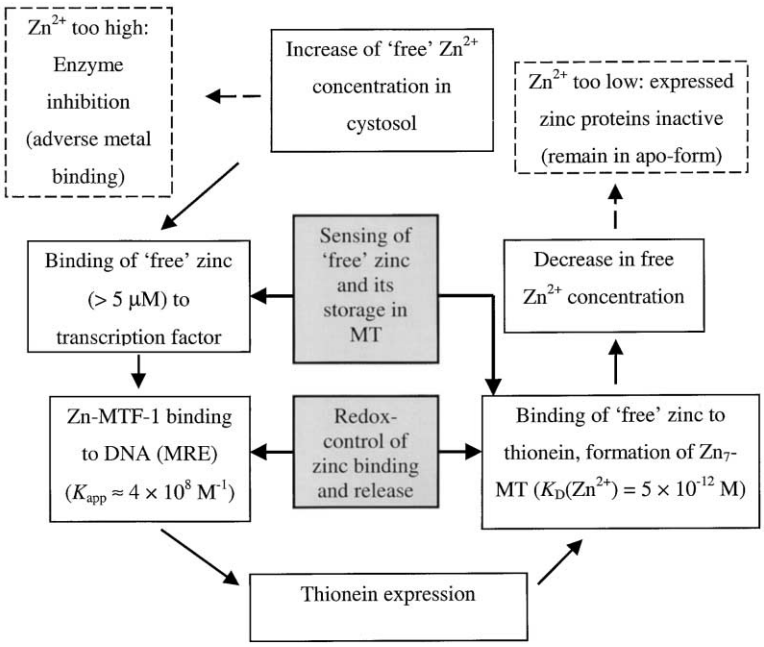

Figure 5. Zinc/Sulfur Proteins and Cellular Zinc Homeostasis

This mechanism is based on recent insights into the function of MTF-1 and MT and should be seen in conjunction with other zinc transport proteins such as ZnT-1 to ZnT-7 [105–109].

MTF-1 DNA binding occurring in the presence of 5 to 15 μM of free zinc ions [114]. MTF-1 therefore only "switches on" once the concentration of unbound cytosolic zinc ions reaches or exceeds low μM concentrations [115]. Below this "free" zinc concentration, the protein is mostly present in its dissociated, metal-free, inactive form. Interestingly, free zinc concentrations in the micromolar range are sufficient to inhibit a range of enzymes, indicating that MTF-1 has an "appropriate" zinc binding constant to act as a zinc sensor. In its active form, MTF-1 initiates the expression of metallothionein I and II genes [116, 117]. Thionein synthesis is therefore triggered in response to excess zinc (or cadmium) ions, providing the cell under "metal stress" with an incredibly strong metal chelator.

Lowering the Concentration of Free Zinc Ions. The exceptionally high thermodynamic stability of MT ($K_D(Zn^{2+}) = 5 \times 10^{-12}$ M [pH 7.4] [118]) means that thionein is a strong biological chelating agent that effectively binds free zinc ions, thereby lowering the free (but not overall) zinc concentration in the cytosol [92, 119–121]. Since thionein is also able to rapidly reactivate enzymes inhibited by zinc, its role in the control of zinc homeostasis has been postulated [92, 119, 122]. This suggestion has recently been supported by studies that have shown unexpectedly high concentrations of thionein in cells [121]. The vast difference in the ability of MTF-1 and MT to bind zinc also backs the role of MTF-1 as zinc sensor, thionein as zinc binding protein, and MT as zinc storage. It is still not clear, however, if and how thionein would remove zinc from MTF-1, a process required to fully close the feedback circuit of zinc regulation.

Zinc Storage and Exchange. The high thermodynamic stability of MT, compared to other zinc proteins (and particularly MTF-1), allows this protein to function in intracellular zinc storage. From a chemical point of view, this high stability is the result of the unique metal/ligand arrangement found in MT. The mammalian protein consists of a single polypeptide chain of approximately 60 amino acids, of which 20 are cysteine residues. Despite the lack of secondary structure, these residues tightly bind seven zinc atoms in a Zn_3Cys_9 and a Zn_4Cys_{11} cluster (see Introduction) [123]. Importantly, these metal ions are kinetically labile and readily participate in intramolecular metal exchange between the clusters [124] and can also transfer zinc to a range of apo-proteins such as the apo-forms of bovine carboxypeptidase A, *E. coli* alkaline phosphatase, sheep liver sorbitol dehydrogenase, rat glutocorticoid receptor fragment, and *Xenopus laevis* transcription factor TFIIIA [119, 122, 125, 126].

Redox Control of Metal Binding. Metal transfer from MT to apo-proteins is rather inefficient, with only about one in seven zinc ions transferred in the absence of mediators such as GSH, citrate, and ATP [119, 125, 127]. Due to the presence of 20 cysteine ligands, the zinc/sulfur clusters are redox sensitive, and ligand oxidation results in zinc release, a process that has been extensively studied in vitro during the last decade [4, 69, 81, 119, 128–131]. Although details of oxidative zinc release in vivo are not yet established, MT readily undergoes thiol/disulfide exchange reactions with numerous oxidants such as cystine, cystamine, selenocystamine, and glutathione disulfide (GSSG) [99, 119, 125, 128, 131]. Since such reactions involve GSSG and are influenced by GSH, a link between MT, zinc homeostasis, and the redox state of the cell has been proposed [122]. The biological implications of this link are not yet fully understood, and MT has been considered as a zinc storage protein [120, 125] as well as a cellular "redox sensor" [132].

The notion of oxidative control of zinc availability within the cell obviously transcends the role of MT. Zinc fingers are known to be sensitive toward oxidation, and oxidative inhibition of MTF-1 activity would also, directly and indirectly, change the availability of zinc ions inside the cell. Since zinc is also known to upregulate the ex-

pression of antioxidant enzymes such as Cu,Zn-superoxide dismutase (Cu,Zn-SOD) [133, 134], oxidative zinc release is a complex process that invokes many different cellular responses, not all of which are yet fully understood in vivo.

Redox Control of Zinc Finger Proteins

Approximately 1% of mammalian genomes encode proteins containing the zinc finger structural motif that is built around a zinc/sulfur complex [135]. The most common class of zinc finger motifs contains zinc ions tetrahedrally coordinated to two cysteine and two histidine residues (e.g., transcription factor SP1), although fingers with metal bound to three cysteine and one histidine residues (e.g., tumor suppressor p53 [136]) or four cysteine residues (e.g., p44 subunit of transcription factor, TFIIH, estrogen receptor) have also been observed [135, 137]. A unique group of zinc finger proteins are the so-called Ring zinc finger proteins that contain two zinc ions bound to cysteine and histidine residues within the same domain, e.g., RING finger proteins SAG, ROC, Rbx, and Hrt [138].

Zinc finger proteins fulfill many biological roles. The finger motif is abundant in many DNA binding proteins, especially transcription factors (e.g., SP1, TFIIIA, NF-kB, MTF-1), where it is a prerequisite for their DNA binding and transcriptional control functions [139]. Oxidation of the cysteine residues leads to the expulsion of the zinc, collapse of the zinc finger structure, and inability of the transcription factor to bind to DNA [135, 140]. Thus, large parts of the cell's gene expression and signal transduction machinery are redox sensitive, which is a circumstance that is also increasingly explored in the context of drug design [135, 139, 141, 142] (see Drug Design).

While it is generally true that cysteine oxidation abolishes metal binding, recent studies have indicated that there might be notable exceptions to this rule. Cysteine sulfenic and sulfinic acid have been found in metalloenzymes such as bacterial nitrile hydratase (NHase), where the sulfur acid binds metals such as iron and cobalt [7, 143]. Oxidized cysteine acids acting as ligands are also being investigated in some transcription factors such as BPV-1 E2 protein and activator protein-1 [39], although the details and precise biochemical role of this sulfur acid-metal coordination is still under discussion. Future studies will without doubt shed more light on the redox control of metal binding to cysteine ligands and its biochemical implications.

Drug Design

The previous sections mentioned examples of metal binding (e.g., MTF-1, thionein) and redox processes (e.g., oxidative regulation, protection of enzymes) that allow cells to respond to changes in their redox environment. The associated area of pharmacological research is large and this review will focus on a few recent advances in redox drugs and metal control. The intention is to show how the issues of redox regulation and metal control, as discussed in Redox Control, Ligand Oxidation and Metal Release, and Regulation of Zinc Homeostasis, have led to pharmacological research with similar biological aims yet a completely different chemistry. These issues, apart from representing important advances in rational drug design, also underline the importance of more fundamental research into cellular redox and metal trafficking pathways.

Redox Drugs

While healthy human cells maintain a highly reducing intracellular redox environment with GSH:GSSG ratios around 100:1 [30, 31], disease formation frequently leads to a significantly disturbed redox balance. Disorders associated with oxidative stress, such as neurodegenerative diseases, aging, inflammation, rheumatoid arthritis, diabetes mellitus, and cancer [144–146], are often characterized by high levels of oxidizing species and an impaired antioxidant defense. In contrast, some disease types exhibit abnormally reducing cellular environments, especially in malignancies such as the RIF-1 murine tumor [147]. These abnormal redox states can be used therapeutically by targeting cells with redox drugs.

There are two main criteria for the design of such redox agents. First, the drug must be either beneficial or inactive to cells with a normal redox balance. Second, it must be either intrinsically toxic to cells with abnormal redox states or, alternatively, it must become activated and thereby exert a toxic effect in response to such a redox environment.

Reductive activation has been utilized to derive selective agents for inducing apoptosis or necrosis in malignant neoplasms. The interior of such a tumor is deprived of oxygen due to unregulated growth without vascular support, creating a hypoxic environment. To overcome this adverse microenvironment, cancer cells must acquire a resistant phenotype, which involves changes in enzyme profile and activity [148]. For example, in vivo DT-diaphorase levels can be increased by as much as 100-fold in lung adenocarcinoma tissue when compared to normal lung tissue, and even higher fold excesses have been reported in vitro for cancer cell lines of the colon, breast, and melanoma [149].

Bioreductive agents take advantage of this changed phenotype to target these cells. Reducing enzymes such as human DT diaphorase, NADH oxidase, and cytochrome *c* reductase show broad substrate specificity. Upon administration of redox-sensitive agents such as mitomycin C and diaziquone (Figure 6) in their oxidized prodrug forms, they are preferentially reduced in cells overexpressing these enzymes. Reduction activates the drug, which can then initiate cytotoxic mechanisms such as DNA strand breakage, DNA crosslinking, and production of radical species [150].

Importantly, not all cancer cells exhibit such an extreme reducing environment. In fact, for some types their redox state can be highly oxidizing. Although most cancer cells are in a hypoxic environment, this does not necessarily imply the environment is also reducing. Recent studies have demonstrated that cells deprived of oxygen may still possess an overall redox state balanced in favor of oxidation, due to the inactivation or downregulation of antioxidant enzymes such as GPx, SOD, and catalase (CAT) and the overactivation of other oxidizing pathways such as fatty acid synthesis [151, 152]. Many cancerous cell types, such as human kidney and prostate, therefore, exhibit what would normally be considered an oxidizing cellular environment [151].

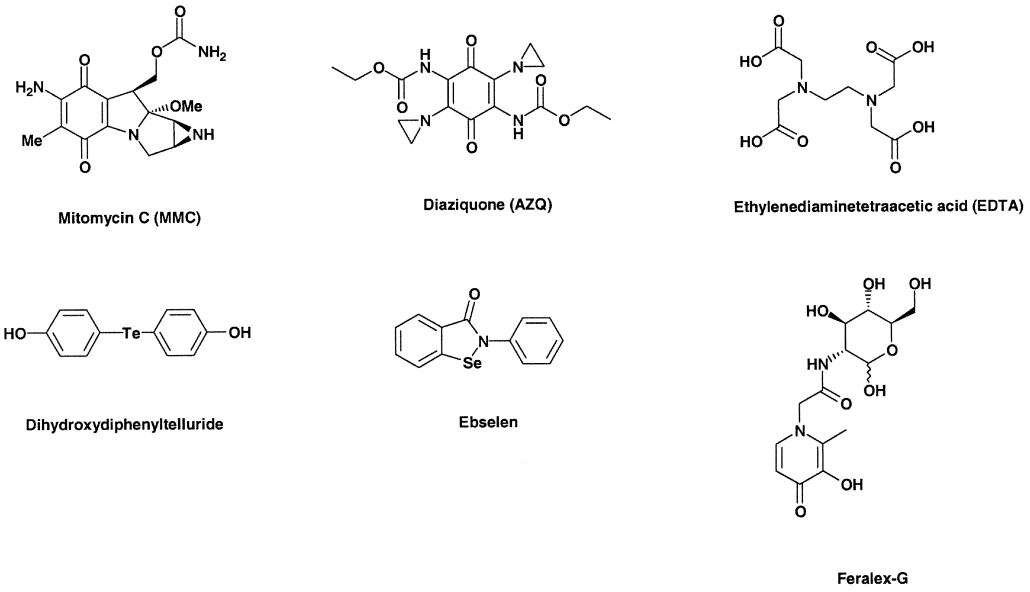

Figure 6. Drugs Designed to Respond to Redox or Metal Imbalances

Bioreductive (Mitomycin C and Diaziquone), catalytic (4,4'-dihydroxydiphenyltelluride and Ebselen), and metal binding (ethylenediaminetetraacetic acid and Feralex-G) compounds that have been proposed to act as therapeutic agents. Details are found in the text.

It has recently been proposed that the administration of small, chemically simple *redox catalysts* that mimic the catalytic cycle of GPx but lack the enzyme's high selectivity for its thiol substrate (primarily GSH) might provide an avenue to utilize this unusually oxidizing redox environment to inactivate cysteine-containing proteins essential for cancer cell proliferation [153]. A range of GPx mimics such as Ebselen and 4,4'-dihydroxydiphenyltelluride (Figure 6) are known to unspecifically catalyze the oxidation of various thiols in the presence of oxidizing agents such as hydrogen peroxide or peroxynitrite [154–156]. In vivo, this might result in a highly effective, catalytic sensitizing of cells with a disturbed redox balance against their own redox environment, thereby inducing "cell suicide" without the need of external stressors such as radiation [153].

Redox catalysis is also an increasingly important area of antioxidant research. Antioxidants are agents that effectively counteract the damaging effects of oxidative stressors. Many antioxidants, such as vitamins A, C, and E, occur naturally and are increasingly used in nutritional supplements and for therapeutic purposes [157–159]. Most natural as well as artificial antioxidants can be classified either as "one shot" antioxidants (reacting with oxidative stressors in stoichiometric ratios) or catalytic antioxidants. Antioxidant enzymes such as SOD, CAT, and GPx frequently provide the blueprint for catalytic antioxidant design, and the therapeutic potential of metal complexes with SOD and CAT activity is currently being evaluated [160].

The design of antioxidants reflects the vicious cycle of metal release and oxidative stressor formation that exists during oxidative stress (Figure 6). Since oxidation of sulfur ligands leads to metal release, adventitious "free" metals not only oxidize cysteine residues but also facilitate hydroxyl radical formation in Fenton-type reactions. This results in a cycle of cysteine oxidation and metal release that is now believed to play a major role during oxidative stress and (neuronal) disease formation [161].

Not surprisingly, control of the unbound cytosolic metal ion concentration provides an alternative to redox control. This has led to a range of possible therapeutic methods, some of which are briefly mentioned in the next section.

Therapeutic Advances Based on Metal Control

The control of the concentrations of different metal ions inside the human body is an important area of pharmacological research. In conjunction with metal supplementation, metal removal has become part of popular medicine and there is a wealth of literature on the use of artificial metal binding agents such as ethylenediaminetetraacetic acid (EDTA, Figure 6) and their potential use in medical therapy [162].

The administration of metal chelators is clinically approved for the treatment of metal poisoning. By competing for the metal ion with biological binding sites, the chelator can effectively remove excess metal. Chelators therefore have two essential characteristics: they must be specific for the metal ion (and sometimes a particular oxidation state) they wish to bind, and they must also have strong binding constants to enable competitive binding. The chelator meso-2,3-dimercaptosuccinic acid (DMSA), for example, is used therapeutically as a drug to reduce blood lead levels in children exposed to environmental contamination [163]. D-penicillamine is clinically used as a copper chelator in the treatment of Wilson's disease, which arises due to a metabolic defect that results in copper deposition [164]. Glutathione and α-lipoic acid have also been proposed as effective chelators for mercury removal [165]. Deferiprone (1,2-dimethyl-3-hydroxypyrid-4-one) has obtained wide-

spread clinical use in the treatment of thalassaemia as a specific iron chelator [166]. A recent advance in chelation therapy has been the development of drugs that strongly bind to more than one metal. Feralex-G (2-Deoxy-2-(*N*-carbamoylmethyl-[*N*'-2′-methyl-3′-hydroxy-pyrid-4'-one])-D-glucopyranose, Figure 6) is a novel chelator for trivalent aluminium and iron that has been proposed for the treatment of Alzheimer's disease. In vitro, the compound has been shown to dissociate these metals from hyperphosphorylated tau proteins [167].

Adventitious iron, copper, and manganese ions have been implicated in the progression of neurodegenerative diseases [144, 161], and the metals have been associated with the presence of oxidative stress in these disorders. Therefore, in addition to competitively chelating these species from proteins, there is also intense interest in removing free metal ions. Agents that strongly bind these redox active metal ions are currently being studied as potential antioxidants, for example in oxidative stress-related skin damage [168]. While the removal of adventitious metal ions must be seen in the context of a range of other reactive species simultaneously present during oxidative stress, chelate administration might supplement antioxidant therapy. In some cases, the metal chelator and redox agent might even be one and the same molecule [169, 170].

Metal removal from metalloproteins can also be achieved in this way. Although the precise role of such an approach, if any, remains unclear, a recent report on the use of metal binding agents in cancer therapy should be briefly mentioned. It has been suggested that agents which can destroy the zinc finger structure of transcription factor SP1, a motif of the protein vital for interaction with DNA (see Regulation of Zinc Homeostasis), have the potential to control viral and cancerous diseases [171]. This can, of course, be achieved in two ways: either by oxidation of the cysteine ligands with subsequent zinc release, or by zinc-chelating agents that remove the zinc from the structural metal site. In both cases, efficiency, specificity, and reversibility of the method play an important role. Although they are still in the very early stages of their development, both leads provide a fertile ground for further biochemical and pharmacological research.

Summary and Outlook

The previous sections have delineated the sheer complexity of cysteine biochemistry by providing a selective overview of cysteine's role in regulation and control of protein activity, which is summarized in Table 1. The three distinct "chemistries" of the amino acid's thiol group, i.e., nucleophilicity, redox activity, and metal binding, provide a powerful combination for a range of important biological control mechanisms ranging from sophisticated cysteine or metal-based regulatory mechanisms to response systems that sense and remove excessive concentrations of free metal ions inside the cell. The wide range of oxidation states of cysteine, its ability to participate in many different redox and metal binding reactions, and the high, millimolar abundance of cysteine thiols in the cell ensure plenty of scope for future research and surprising discoveries in the field of cysteine biochemistry. Recent excitement in peroxiredoxin research is a good example of this [172–174].

Considering the understanding of regulation and cellular signaling, redox and metal control of cysteine is likely to play an increasingly important role, and there are several chemically possible regulation mechanisms that have not yet been identified in proteins. These include sulfur oxidation states less studied in a biological context, such as sulfenic and sulfinic acids, and the oxidative formation of "unusual" metal binding sites.

The increasing knowledge of cysteine redox transformations and how they influence metal binding also has pronounced effects on the design of future generations of drugs. Redox transformations are important in many human disorders, among them diseases related to oxidative stress. The design of effective antioxidant drugs is one such promising avenue to control some of these problems. In addition, a better understanding of how cellular redox processes work also paves the way to utilize redox or metal imbalances in sick cells to gain a degree of drug specificity not available at present. With cysteine proteins playing a major role in cell proliferation, health, and death, this chemistry is likely to be at the heart of many biochemical and pharmacological investigations in the future.

Acknowledgments

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