Comments on Inorganic Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713455155

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To cite this Article Otvos, James D. , Petering, David H. and Shaw, C. Frank(1989) 'Structure—Reactivity Relationships of Metallothionein, a Unique Metal-Binding Protein', Comments on Inorganic Chemistry, 9: 1, 1 — 35
To link to this Article: DOI: 10.1080/02603598908035801
URL: http://dx.doi.org/10.1080/02603598908035801

Structure–Reactivity Relationships of Metallothionein, a Unique Metal-Binding Protein

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Metallothionein is an intensively studied protein, which binds a variety of essential, toxic, and pharmacologically active metals, including Zn, Cd, Au, and Pt. Until recently, attention focused primarily on its biological properties and static features of its chemistry. It is now apparent that metallothionein is a remarkably reactive protein in metal exchange and ligand substitution reactions and in its interactions with a number of electrophilic compounds, which are both metallic and organic in nature. This unique behavior finds its basis in the dynamic character of its metal–ligand structure, which is sensitively probed by ^{113}Cd NMR techniques. In an effort to relate the chemistry of metallothionein to its cellular activities, it is shown that the kinetic reactivity of the metal binding sites of metallothionein distinguishes it from other typical metalloproteins involved in enzyme catalysis. The rich inorganic chemistry of this structure is clearly important for some of its known functions and is suggestive of other novel roles for this protein in cells.

Key Words: *zinc, cadmium, gold, platinum, metal metabolism, metal toxicity, metallodrugs, metallothionein*

INTRODUCTION

Metallothionein is a remarkable metal-binding protein found in mammals and other organisms, which has attracted intense and

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Comments Inorg. Chem.
1989, Vol. 9, No. 1, pp. 1–35
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sustained scrutiny since its discovery three decades ago.[1] First examined as a structure involved in the metabolism of the toxic metal cadmium, subsequent studies demonstrated its involvement in the reactions of excessive concentrations of other heavy metals such as mercury with liver and kidney.[2,3] Still later it became apparent that metallothionein (MT) plays conspicuous roles in the normal, physiological metabolism of the biologically essential metals, zinc and copper.[4,5] Thus, among metalloproteins, the metal composition and activities of metallothionein is uniquely a function of the previous history of the organism and cells of origin of the protein.

One of the properties of this protein which has been repeatedly examined is the induction of its synthesis in cells exposed to elevated concentrations of Cd^{2+}, Zn^{2+}, or Cu^{2+}.[6-8] The response is rapid and efficient; it leads to the sequestration of most of the metals entering such cells by metallothionein and appears to protect them from heavy metal toxicity.[9] Such results have led to the view that MT is basically a detoxification protein (Fig. 1). The scheme accommodates the facts that (A) these metals cause the synthesis of new messenger RNA for the *de novo* synthesis of apo-MT (metal-free) protein,[10] that (B) the protein binds excess intracellular metals, and that (C) the metalloprotein has a finite lifetime and is biodegraded (hydrolyzed) by proteases with the release of bound metals.[11,12] In the case of cadmium, the metal rebinds to apo-MT to establish a steady state concentration of the cadmium protein in cells.[11]

Most cells have detectable, basal levels of metallothionein in the absence of heavy metal induction.[13] High levels are found, for example, in mammalian fetal liver, which may need to store nutrient metals for the host, and in the livers of freshwater fishes throughout their lifetimes.[14,15] Furthermore, hepatic zinc metallothionein induction also accompanies numerous stresses unrelated to metal exposure, such as infection and other trauma, during which substantial redistribution of Zn, Cu, and Fe takes place between plasma and liver.[16,17] One interpretation of these findings is that MT behaves as a transient storage site for these metals, buffering the cell from changing influxes of metals, which otherwise might be toxic (Fig. 1 with Zn or Cu substituting for Cd). According to this view, MT fulfills a similar role in essential as well as toxic metal metabolism. The precedent for this picture is the current

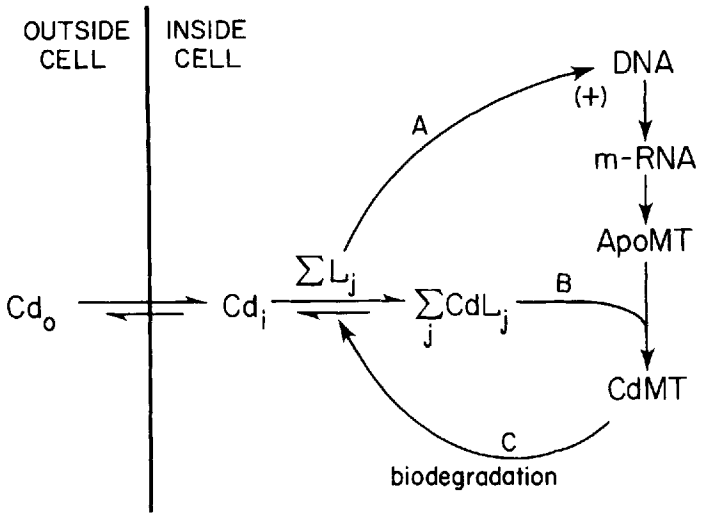

FIGURE 1 Simple diagram of Cd distribution in cells. L represents pool of intracellular ligands other than apo (metal-free) MT, which can bind Cd.

understanding of the role played by ferritin in storing excess iron entering various tissues.[18]

In this model MT chelates metals in an essentially irreversible step to form chemically inert products, which do not interact with other intracellular components. Only upon hydrolysis of the protein structure do bound metals become available for possible reaction with the rest of the cell. The basic input of chemical information undergirding this picture comes from two early papers by Vallee and co-workers.[19,20] Then, until the 1980's, there was little research conducted on the structure and reactivity of metallothionein. With the investigations of the past decade, it is now clear that the protein is a remarkably reactive chemical structure, whose dynamic properties need to be integrated into the understanding of the biological functions of the molecule. The comments which follow set forth our results in support of an understanding that the chemistry of zinc and cadmium metallothionein in cells may be much more elaborate than previously thought.

OVERALL STRUCTURE OF METALLOTHIONEIN

The metallothionein protein in mammals contains 61 or 62 amino acids, 20 of which are cysteine residues located in strictly conserved positions in the amino acid sequence. The cysteinyl thiolate side chains function as the sole ligands for the complexation of metal ions.[21] Zinc and cadmium bind with a stoichiometry of seven metal ions per protein molecule, while as many as twelve Cu^{1+} ions may complex with the protein.[22] Interestingly, hepatic and kidney metallothioneins are frequently isolated as mixed-metal species, containing an assortment of metals such as Zn, Cu, and Cd or other heavy metals, depending on the history and physiological state of the organism.[23] Indeed, although Cd binds about 10^3 times more strongly to the protein than Zn (see below), hepatic MT is always isolated as a Cd,Zn-protein after exposure of the animal to cadmium.[24]

NMR studies with ^{113}Cd-MT first demonstrated that the metals in metallothionein exist in two metal clusters (see below).[25] Since that time, both X-ray crystallographic analysis of Cd_5, Zn_2-MT and NMR determination of the solution structure of Cd_7-MT have confirmed that the polypeptide chain wraps sequentially around each metal cluster, generating two independent, globular domains.[26,27] From this work it is apparent that the entire protein is efficiently organized to bind multiple metals and does not have excess polypeptide *to bury* the metal sites within a larger protein structure as is commonly the case with other metalloproteins. On this basis alone one might suspect that metallothionein would be generally more reactive in reactions involving the clusters—metals and thiolate ligands—than other structures.

FORMATION AND STRUCTURAL PROPERTIES OF HOMOGENEOUS METAL-METALLOTHIONEINS AND MIXED-METAL METALLOTHIONEINS

The current understanding of the mode of Cd and Zn binding to MT has been provided by numerous studies during the past decade using a wide variety of spectroscopic techniques.[28,29] Among them, ^{113}Cd NMR has perhaps been the most informative because of the

extreme sensitivity of the Cd chemical shift to subtle differences in coordination environment, which makes it possible to distinguish separate resonances from ^{113}Cd bound to each of the 7 binding sites of $^{113}Cd_7$-MT. One method to prepare this species is to displace zinc with cadmium from naturally occurring Zn_7-MT,

$$Zn_7\text{-MT} + 7^{113}Cd^{2+} \rightleftarrows {}^{113}Cd_7\text{-MT} + 7Zn^{2+} \qquad (1)$$

This metal exchange reaction goes to completion under stoichiometric conditions and is finished within seconds.

Figure 2A shows a ^{113}Cd NMR spectrum of the rabbit liver ^{113}Cd-MT-2 isoprotein (rabbits and other mammals produce several isoforms of metallothioneins, differing slightly in amino acid sequence). Analysis of the 2-bond ^{113}Cd-^{113}Cd spin coupling connectivities responsible for the multiplet splitting patterns of each resonance indicates that MT contains 2 metal-thiolate clusters, one with 3 metals (Cd_3Cys_9) and the other containing 4 (Cd_4Cys_{11}).[25,30] As shown in the schematic cluster structures in Fig. 2A, 8 of the 20 cysteine sulfurs serve as bridging ligands between adjacent metal ions, with the remaining 12 cysteines providing terminal ligation to complete the tetrathiolate coordination of each Cd. Selective proteolysis studies subsequently showed that the cysteine ligands of each cluster are contiguous in the amino acid sequence so that the protein is divided into two segments, the amino-terminal β-domain enfolding the 3-metal cluster and the carboxy-terminal α-domain containing the 4-metal cluster.[31]

Two-dimensional ^1H NMR studies by Wüthrich and co-workers provided the next higher order of NMR structural analysis. The first step was to correlate the chemical shifts of the β-CH_2 protons of each cysteine residue with the particular Cd^{113} ion to which it is coordinated. This was accomplished using heteronuclear multiple quantum chemical shift correlation experiments which exploit the existence of 3-bond scalar coupling between the cysteine ligand protons and the spin-1/2 metal ions.[32] This information, in conjunction with the sequence-specific proton resonance assignments made for the entire protein by ^1H COSY and NOESY experiments, permitted unequivocal identification of the amino acid sequence positions of the cysteine residues ligated to each Cd.[26,33] With these results and additional pairwise proton distance constraints gener-

ated from ^{1}H NOESY spectra, distance geometry calculations were carried out to determine the three-dimensional solution structure of each cluster domain.[26] The spatial relationship between the protein domains was not defined because of lack of interdomain NOESY information. This is suggestive of a relative structural independence of the two parts of the metallothionein molecule also seen in the X-ray structure.[27]

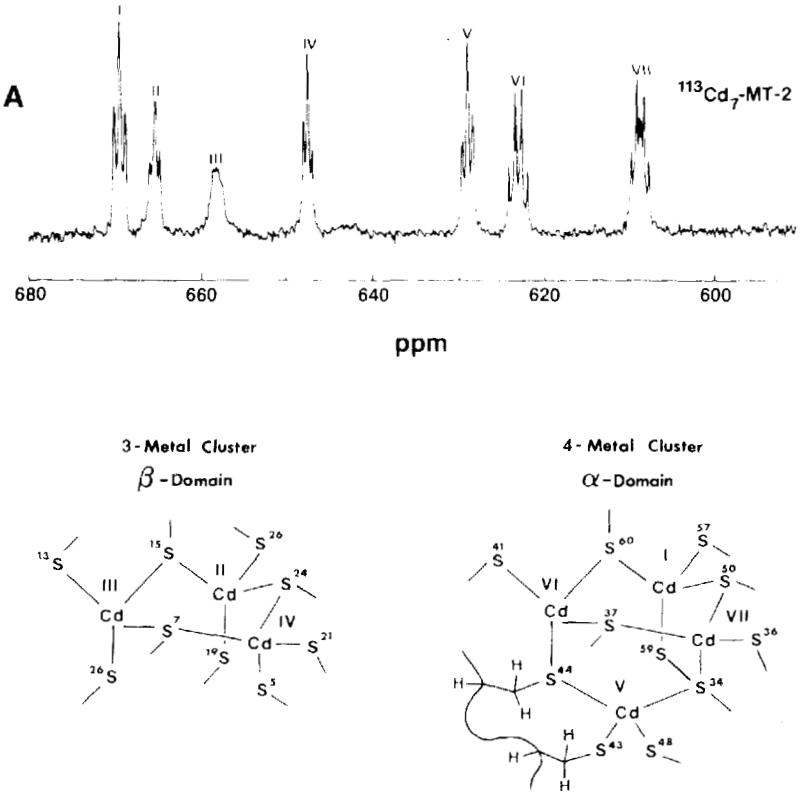

FIGURE 2 (A) ^{113}Cd NMR spectrum at 40° of rabbit liver Cd_7-MT-2 and schematic representation of the structures of the metal clusters. The Roman numerals identify the Cd ions giving rise to each resonance in the spectrum and the numbers identify the positions of the cysteine ligands in the protein sequence. (B) ^{113}Cd NMR spectrum of native rabbit liver Cd,Zn-metallothionein-2 and schematic structures of the metal compositions of the clusters in this heterogeneous mixture of species.

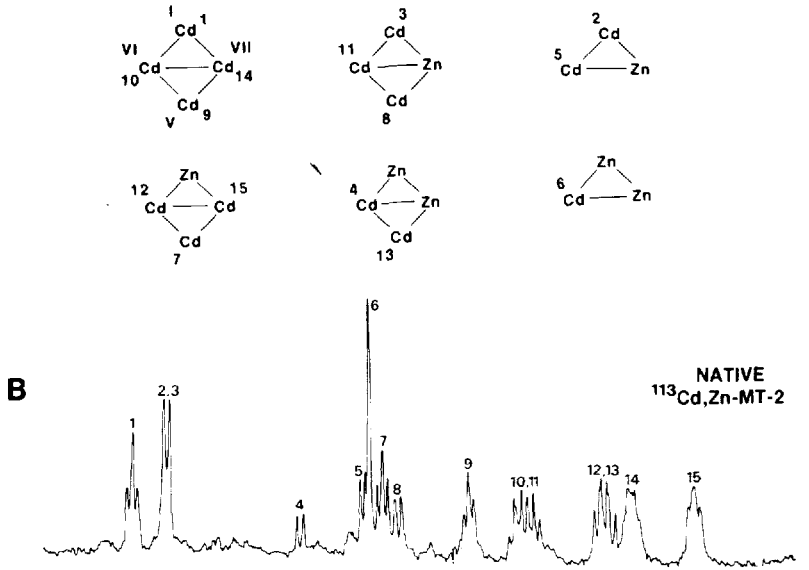

FIGURE 2 (continued)

Intriguingly, the NMR-derived structure is substantially different than the X-ray structure of native rat liver Cd_5, Zn_2-MT-2.[27] Although agreeing on the gross features of metal coordination to the 2 clusters, the structures differ in the identification of the specific cysteine residues which serve as ligands to the metals. Recent NMR experiments on the same rat liver protein used in the X-ray studies indicate that the discrepancy is not due to differences in species origin of MT or to the method of acquisition of the Cd-protein.[34]

Some of the differences might be rationalized if metallothionein has the ability to adopt, via intramolecular thiolate ligand exchange, two or more configurational states, which possibly exhibit preferential affinity for clusters of different metal composition and site occupancy. Support for the idea that MT in solution interconverts continuously between two or more configurational states comes from the marked temperature dependences of the linewidths of several of the ^{113}Cd resonances of the protein. Lowering the tem-

perature from 40° to 5° induces chemical exchange broadening of the signals from all three Cd ions in the 3-metal cluster (resonances 2–4), especially that of resonance 3.[35] Further decrease in temperature to −30° in a 30% methanol solution induces the additional broadening of resonance 7 of the 4-metal cluster. Consequently, at room temperature it appears that the metals in both clusters are in rapid interchange between two or more *states*, which differ in local metal environment. As yet, there is no direct proof that these are configurational states which interconvert via cysteine thiolate ligand exchange, though it is a reasonable assumption based on the reported lability of the metal-thiolate bonds in model inorganic cage complexes of similar structure.[36]

Not only is it likely that certain cysteine ligands exchange rapidly between different metals, the metal ions themselves move rapidly between different binding sites in the protein. This was first shown by ^{113}Cd NMR saturation transfer experiments, which demonstrated that the Cd ions interchange between sites in the 3-metal cluster much faster than they move between sites of the 4-metal cluster or between sites in different clusters.[37] Surprisingly, the data indicate that the lifetime of Cd in a particular 3-metal cluster site is only about 0.5 seconds at 30°. The mechanism for this facile intersite metal exchange process might plausibly be intramolecular, intermolecular, or both. Recent experiments, in which saturation was observed to be transferred from Cd in the 3-metal cluster of one MT isoform to 3-metal cluster sites in another isoform, provide direct proof that intermolecular metal transfer is occurring on this rapid timescale. The possibility of concurrent intramolecular metal exchange cannot yet be ruled out. These unexpected results show MT to be a remarkably fluctional molecule.

The physiological importance of interprotein metal exchange reactions of MT was demonstrated in NMR studies of the Cd and Zn distribution among the multiple sites in the mixed-metal protein produced *in vivo* by Cd induction.[30,35] By analyzing the chemical shifts and spin-coupling connectivities of the 15 resonances appearing in the ^{113}Cd spectrum of this material, it was deduced that the native protein is a heterogeneous mixture of species containing clusters of different metal composition (Fig. 2B). What is noteworthy is that the same Cd NMR spectral "fingerprint" of metal distribution is observed after both short- and long-term exposure of rabbits to Cd by injection. Thus, there appears to be a driving

force *in vivo* for the production of a characteristic steady-state distribution of MT species in which Cd and Zn are partitioned preferentially into 4-metal and 3-metal clusters, respectively. Strong evidence exists that this is achieved via a direct metal interchange reaction between Cd_7-MT and Zn_7-MT,

$$m\text{Cd}_7\text{-MT} + n\text{Zn}_7\text{-MT} \rightleftarrows (m+n)\text{Cd}_{7m/(m+n)}, \text{Zn}_{7n/(m+n)}\text{-MT} \quad (2)$$

since it is only by this route and not by reaction 3

$$\text{Zn}_7\text{-MT} + m\text{Cd}^{2+} \rightleftarrows \text{Cd}_m,\text{Zn}_{7-m}\text{-MT} + m\text{Zn}^{2+} \quad (3)$$

that protein can be prepared *in vitro* which gives a ^{113}Cd spectrum identical to that of native Cd,Zn-metallothionein.[35] In fact, a whole series of mixed-metal metallothioneins is produced by this reaction depending on the values of m and n, which are different from those generated in Eq. (3).

These NMR results characterize the metal-thiolate clusters of MT as surprisingly reactive metalloprotein structures. Indeed, even the simple, rapid exchange of metals summarized in Eq. (1) is distinctly different from the behavior of some other Zn-metalloproteins which have been examined (Table I). For example, with Zn-carboxypeptidase metal exchange takes many hours. With three competing metal ions (Cd, Hg, Co) the first order rate constants of 0.03–0.07 hr^{-1} resemble a measured dissociation rate constant for Zn-Cp of 0.024 hr^{-1}, suggesting that exchange rate is controlled by the dissociation of metal from the protein.[38,39] Similarly, metal-exchange processes in superoxide dismutase are qualitatively slow[40,41]; self-exchange of Zn or exchange of Zn for other metals in carbonic anhydrase is not observed.[42] In each case, once a particular metal has entered a protein metal-binding site, it is only slowly replaced by other metal ions.

LIGAND SUBSTITUTION CHEMISTRY AND APPARENT STABILITY CONSTANTS OF Zn_7- AND Cd_7-METALLOTHIONEIN

Two studies published in 1980 demonstrated that the metals in Zn_7-MT are reactive in ligand substitution reactions involving EDTA

TABLE I

Metal exchange reactions of Zn-proteins

Carboxypeptidase (Cp)[38,39]		
$Zn\text{-}Cp + M^{2+} \rightleftarrows M\text{-}Cp + Zn^{2+}$	$k = 0.03\ hr^{-1}$	M = Cd, Hg conditions: 0.1 mM Zn-Cp, M^{2+}
	$k = 0.07\ hr^{-1}$	M = Co; 0.1 mM Zn-Cp, 10 mM M^{2+}
$Co\text{-}Cp + Zn^{2+} \rightleftarrows Zn\text{-}Cp + Co^{2+}$	$k = 0.2\ hr^{-1}$	0.1 mM Co-Cp, Zn^{2+}
Cu_2, Zn_2-Superoxide Dismutase (SOD)[40,41]		
$Cu_2, Cu_2\text{-}SOD + 2Zn^{2+} \rightleftarrows Cu_2,Zn_2\text{-}SOD + 2Cu^{2+}$		slow reaction
$Cu_2, Zn_2\text{-}SOD + 2^{64}Cu^{2+} \rightleftarrows {}^{64}Cu_2,Zn_2\text{-}SOD + 2Cu^{2+}$		slow
$Cu_2, Zn_2\text{-}SOD + 2^{65}Zn^{2+} \rightleftarrows Cu_2,{}^{65}Zn_2\text{-}SOD + 2Zn^{2+}$		slow
Carbonic Anhydrase (CA)[42]		
$M\text{-}CA + {}^{65}Zn \rightleftarrows {}^{65}Zn\text{-}CA + M$ M = Zn,Co,Ni,Cu,Cd,Hg	exchange inert	
Metallothionein[35,37]		
$Zn_7\text{-}MT + nCd^{2+} \rightleftarrows Zn_{7-n},Cd_n\text{-}MT + nZn^{2+}$		fast (halftime, secs)
$^{113}Cd_7\text{-}MT + nCd^{2+} \rightleftarrows {}^{113}Cd_{7-n},Cd_n\text{-}MT + n{}^{113}Cd^{2+}$		rapid
$nCd_7\text{-}MT + mZn_7\text{-}MT \rightleftarrows (m+n)Cd_{\frac{7n}{m+n}},Zn_{\frac{7m}{m+n}}\text{-}MT$		rapid (halftime, mins)

and a number of apo-Zn metalloproteins (Zn-free) competing ligands (L) (reaction 4).[43,44]

$$Zn_7\text{-MT} + 7L \rightleftarrows 7Zn\text{-}L + MT \quad (4)$$

Since that time Zn_7-MT has been shown to react with a variety of small molecular weight ligands (Table II). These ligands fall into three categories of structures and display a range of reactivity with the protein.

Some, like H_2KTSM_2, terpyridyl, and NTA, display biphasic kinetics in the metal exchange process. Among these one observes both ligand-independent and ligand-dependent kinetic behavior. Focusing on H_2KTSM_2, each step produces and comprises about 50% of the total reaction.[45] The rate expression for each kinetic class of metal is first order in zinc and independent of competing ligand concentration (Table II). When the kinetics of reaction of two successive increments of H_2KTSM_2 with Zn-metallothionein are examined, both have the same quantitative, biphasic character, independent of the amount of metal removed from the protein. This suggests that there are two kinetic classes of metals in the protein, which, in effect, react cooperatively with this ligand. Alternatively, there are seven independent metal-binding sites of two kinetic types, each demonstrating uniform ligand substitution behavior no matter how many metals are bound to the protein.

The concept of cooperative kinetic classes of metals in Zn_7-MT is further supported by calculations of the stability constants for metals in Zn-metallothionein.[45] Using H_2KTSM_2 and NTA as competing ligands of known thermodynamic stability with Zn, it has been shown that the same apparent stability constants per Zn in MT are defined whether the metals are grouped in two kinetic classes or are treated as seven non-interacting sites of equal affinity for Zn^{2+}. The results are independent of the amount of metal removed from the protein pool during these reactions, implying that only fully occupied or empty protein binding sites are present at equilibrium. From this analysis all seven zinc ions bind to metallothionein with indistinguishable apparent stability constants of $1.8 \pm 0.6 \times 10^{11}$ per Zn at pH 7.4 and 25° in 0.1 M KCl.

It is natural to identify the kinetic classes with the two metal clusters of the protein. Considering the structure of these clusters,

TABLE II

Ligand substitution reactions of Zn_7-MT

Competing Ligand	Properties of Reactions[a]
3-ethoxy-2-oxobutyraldehyde bis (thiosemicarbazone) (H_2KTSM_2)[45,46]	biphasic: $v = (k_{fast} + k_{slow})[Zn]_{total}$ $k_f = 1.6 \times 10^{-3}$ sec^{-1}, $k_s = 1.2 \times 10^{-4}$ sec^{-1}
Nitrilotriacetate[46]	biphasic reaction: $k_s = 5.1 \times 10^{-4}$ sec^{-1}
Pyridylazoresorcinol (PAR)[47]	one component: $v = (k_{1f}[PAR] + k_{2f})[Zn]_{total}$ $k_{1f} = 3.8 \times 10^{-2}$ M^{-1}sec^{-1}, $k_{2f} = 8.5 \times 10^{-6}$ sec^{-1}
Terpyridyl (TP)[46]	biphasic: $v = (k_{1f}[TP] + k_{2f} + k_{1s}[TP] + k_{2s})[Zn]_{total}$, $k_{1f} = 1.7$ M^{-1}sec^{-1}, $k_{2f} = 1.1 \times 10^{-3}$ sec^{-1}, $k_{1s} = 8 \times 10^{-2}$ M^{-1}sec^{-1}, $k_{2s} = 6 \times 10^{-5}$ sec^{-1}
EDTA[13,46]	one component: Zn-MT + EDTA $\overset{K}{\rightleftharpoons}$ EDTA-Zn-MT $\overset{k}{\longrightarrow}$ Zn-EDTA + MT $K = 800$ M^{-1}, $k = 1.3 \times 10^{-2}$ sec^{-1}
2,2,2-triaminotriethylamine[46]	reactive
Triethylenetetramine, bipyridyl, histidine, cysteine[46]	unreactive
Apocarbonic anhydrase (apo-CA)[43]	$v = k$[apo-CA][$Zn]_{total}$, $k = 1.5 \times 10^3$ M^{-1}sec^{-1}
Apoalkaline phosphatase, apothermolysin, apoaldolase[44]	reactive

[a]Conditions: 25°, pH 7.4, rat liver Zn_7-MT-1 and/or Zn_7-MT-2 (isoproteins).

particularly the architecture of bridging ligands linking individual metals (Fig. 2), it would not be surprising if each cluster behaves as a cooperative unit, not as a set of independent metal–ligand sites. In this interpretation, kinetic constants for ligand substitution would represent rate limiting events, which open up entire clusters to further, rapid reaction with competing ligands. Furthermore, the uniformity of the apparent stability constants per zinc would also argue that entire clusters form and break down without stable intermediates having partial metal occupancy.

Metallothionein has an abundance of thiolates, which potentially could bind substoichiometric amounts of metal at individual sites (Eqs. (5) and (6), for the binding of Zn^{2+} to the β domain thiolate ligands).

$$Zn^{2+} + C_\beta \overset{K_1}{\rightleftarrows} Zn\text{-}C_\beta \tag{5}$$

$$Zn^{2+} + Zn\text{-}C_\beta \overset{K_2}{\rightleftarrows} Zn_2\text{-}C_\beta \tag{6}$$

$$Zn^{2+} + Zn_2\text{-}C_\beta \overset{K_3}{\rightleftarrows} Zn_3\text{-}C_\beta \tag{7}$$

The lack of such species suggests that the cluster structures (e.g., Zn_3-C_β) are favored such that $K_3 >> K_1, K_2$, possibly because the energetic requirements for protein folding around them is minimized in comparison with the formation of other fragmentary structures.

Some competing ligands react with Zn_7-MT with single step kinetics. The rate law for the reaction of Zn-metallothionein with PAR contains both first and second order terms, the latter being first order in both Zn and PAR. The first order process has a rate constant of 8×10^{-6} sec^{-1}, which is nearly two orders of magnitude less than the smaller of the two first order constants for H_2KTSM_2. Clearly, the dissociative kinetic processes with these two ligands involve different rate limiting molecular events. For example, the faster reactions of the bis(thiosemicarbazone) with metallothionein might represent a rate determining bond-cleavage within clusters without loss of metal to initiate this reaction (Fig. 3A), whereas the first order component of the reaction of PAR with Zn_7-MT may involve additional, slower dissociation of thiolate ligands followed by rapid chelation of the metal by PAR. First

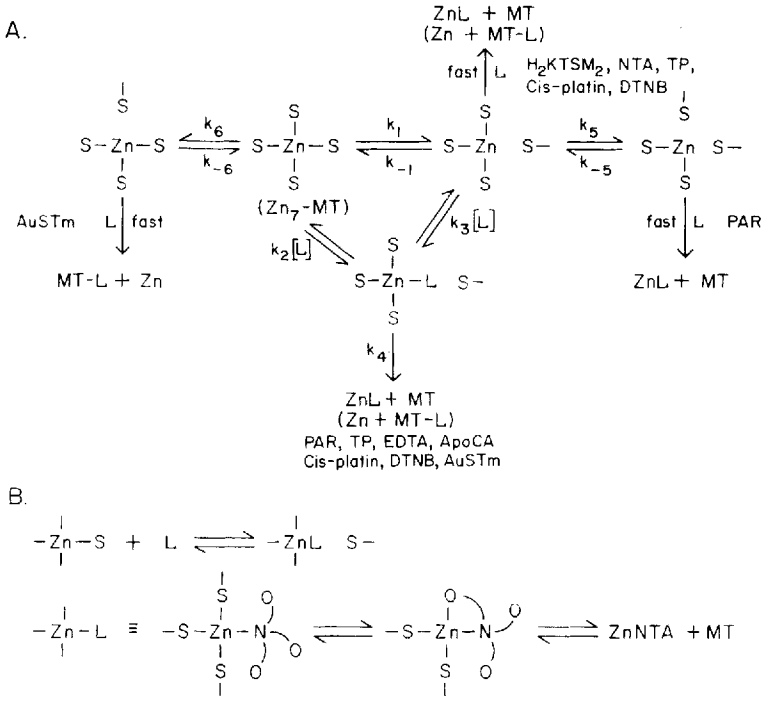

FIGURE 3 Mechanisms of reaction of Zn_7-MT. (A) Pathways for ligand substitution and reactions of metal-coordinated thiolate ligands. L is generic symbol for reagents reacting with clusters as listed near the products of the various paths. It is assumed that the rate-limiting chemistry of these reactions involves particular tetrathiolate metal-binding sites in the clusters. (B) Detail of reaction of intermediate metal–ligand adduct in ligand substitution reactions.

order displacement of metals from metallothionein by Au(I)-thiomalate (described below), which is more rapid than the reaction of H_2KTSM_2 with Zn_7-MT, supports the view that there may be several dissociative mechanistic routes by which reagents can react with the two clusters.[47]

A reexamination of the reaction of EDTA with Zn_7-MT as well as related ones with other polyaminocarboxylates shows that ligand substitution is preceded by a binding step (Table II). Presumably, the kinetics reflect an initial attack of these ligands upon one or both of the clusters, leading to a protein-bound intermediate, which

reacts further to complete the ligand exchange process (Fig. 3). Interestingly, the observation of single step kinetics with several ligands suggests that both clusters react with them according to similar mechanisms and quantitative rate constants. The alternative, that depopulation of one cluster (C_α or C_β) causes a rapid redistribution of metals from the other cluster into the first one cannot be excluded (reaction 8).

$$4Zn_3\text{-}C_\beta + 3C_\alpha \rightleftarrows 3Zn_4\text{-}C_\alpha + 4C_\beta \quad (8)$$

However, the biphasic reactions approaching equilibrium, described above, give no indication that such chemistry occurs when metal-free domains are present.

At large concentrations of competing ligand, NTA and Cd_7-MT react in a biphasic manner to establish equilibria as in reaction 4, from which apparent stability constants for Cd_7-MT can be extracted.[45] As with the Zn protein, the two kinetic classes of metals observed with the ligand substitution process have the same apparent stability constants within error, $4 \pm 2 \times 10^{14}$ per Cd. Thus, as found with many thiolate ligands, MT binds Cd with higher affinity than Zn.[48]

The conclusion that log apparent stability constants for the two clusters for Zn^{2+} (11.2) or for Cd^{2+} (14.6) are the same within error complicates the understanding of the energetics underlying reaction 2, in which Cd from 3-metal clusters tends to exchange with Zn in 4-metal clusters. If each cluster binds either metal with exactly the same apparent stability constant, no energetic advantage can be obtained from the exchange of metals between the two clusters apart from a small, favorable entropy of mixing, which does not account for this metal exchange phenomenon. One explanation is that the driving force for this reaction is actually the formation of mixed-metal clusters among the manifold species generated in the reaction, which are more stable than homogeneous metal clusters. Another is that within the range of the experimental measurements, there is enough error to permit differentials in apparent stability constants of the Zn- and Cd-clusters significant enough to account for the preferential movement of Cd from 3-metal into 4-metal clusters.[45]

That there is some difference in the apparent stability of the two

clusters is suggested by experiments in which apo-MT is titrated with metals.[49] According to this work, metals cooperatively populate the 4-metal cluster first, arguing that reaction 8 is, indeed, favorable. Even so, for this to be important for reaction 2, the ratio of stability constants per Cd for each cluster ($K_{4\text{-metal}}/K_{3\text{-metal}}$) must be larger than the ratio for Zn in order to favor the reaction.

Finally, properties of the reaction of Zn_7-MT with a metal-free (apo) Zn protein, apo-carbonic anhydrase (apo-CA) have been examined.[43] With the log apparent stability constant of Zn-carbonic anhydrase given as 12 at pH 7, this reaction proceeds to completion under stoichiometric conditions, yielding the enzymatically active Zn-protein.[50] What is particularly interesting about this process is that it occurs orders of magnitude faster than any of the substitution reactions involving low molecular weight ligands and at rates comparable to the reconstitution of apo-CA with Zn^{2+} (Table II and Ref. 51). Under the conditions investigated, this reaction is first order in Zn and apo-protein. According to temperature dependence studies, the $\Delta H^{\ddagger}$ is +7 kcal/mol and $\Delta S^{\ddagger}$ is −22 entropy units.[43] Our interpretation is that most of the kinetic barrier to reaction involves orienting to the two macromolecules to undergo productive ligand substitution; less of it involves bond making or breaking.

Although the mechanistic details are not yet in hand, it is striking that metal transfer occurs so readily between two protein structures. It is even more so when this result is compared with the reconstitution of metal-free carbonic anhydrase with Co^{2+} provided by various ligands.[52] In these reactions it is found that only coordinatively unsaturated cobalt complexes react efficiently with the metal-acceptor protein, whereas with metallothionein fully coordinated metal ions in metal clusters are kinetically accessible to carbonic anhydrase.

One can rationalize the diverse kinetic results described in this section by assuming that there are associative and dissociative paths of reaction available to various ligands as shown in Fig. 3A. For simplicity and because it appears that unsaturated clusters are unstable (reactions 5–7), it is hypothesized that the removal of the first metal ion is rate-limiting for depopulation of an entire cluster. Then, depending on the nature of L (competing ligands or other reagents examined below), we envision either direct bimolecular

reactions (k_2) or a combination of two processes (k_1 and k_3), which produce intermediates that undergo rate limiting breakdown (k_4), or rate determining, unimolecular processes (k_1, k_5, or k_6) such as a metal-sulfhydryl bond dissociation, which are followed by rapid conversion to product in the presence of L.

The dissociative events do not produce free metal ion before attack by L, for even under thermodynamically favorable conditions only some ligands effectively compete for zinc. Perhaps, as shown in Fig. 3B, at least three donor atoms from a polydentate ligand need to displace thiolates before product can be formed. Indeed, there may be steric requirements for such reactions. Thus, the tripod polyamine, 2,2′,2″-triaminotriethylamine, but not the linear triethylenetetramine, reacts with Zn-metallothionein.

The kinetic properties of Zn_7-MT in ligand-substitution reactions may be compared with those of other Zn-metalloproteins (Table III). Chelating agents can remove Zn from metalloproteins other than metallothionein. However, the results for carboxypeptidase, carbonic anhydrase, and superoxide dismutase show that these structures differ from MT in their mechanisms of reaction. Whereas a variety of multidentate ligands can react with Zn_7-MT by associative and dissociative pathways, which do not involve free Zn^{2+} as an intermediate (Fig. 3), EDTA extracts zinc from carboxypeptidase in a reaction limited by the rate of dissociation of Zn^{2+} from the enzyme and does not react at all with carbonic anhydrase or superoxide dismutase at pH 7. Only bidentate and a few tridentate ligands can bind to carboxypeptidase or carbonic anhydrase prior to ligand substitution. In contrast, Zn_7-MT appears to be inert to substitution by bidentate ligands but reacts with a variety of multidentate ligands.

SULFHYDRYL REACTIVITY OF METALLOTHIONEIN

The metal-coordinated thiolates of MT are also reactive centers, which generally interact with electrophilic agents. Such reactions have been documented *in vivo*, in cell culture and in chemical experiments. Postulated biological activities that involve the thiolates include detoxification of CCl_4,[56] reactions with organic alkylating agents such as chlorambucil[57] and iodoacetate,[56] inter-

TABLE III

Comparative ligand substitution chemistry of Zn-proteins

Protein	Properties of Reaction
Carboxypeptidase (pH 7)[53,54]	k
L:2,6-dipicolinic acid	3 $M^{-1}sec^{-1}$
2,2′-bipyridyl	13
8-hydroxylquinoline-5-sulfonate	75
1,10-phenanthroline	275
NTA	$4 \times 10^{+4}$ sec^{-1}
EDTA	$4 \times 10^{+4}$
Carbonic anhydrase (pH 5)[55]	
2,6-dipicolinic acid	0.3 $M^{-1}sec^{-1}$
1,10-phenanthroline	0.001
EDTA, NTA	2×10^{-5} sec^{-1}
Cu,Zn-Superoxide Dismutase[40,41]	
EDTA, 1,10-phenantholine	unreactive

actions with electrophilic metallodrugs including gold anti-arthritics[58] and platinum anti-tumor agents,[59,60] and scavenging of superoxide and hydroxyl radicals.[61] In this section we will address primarily the chemical studies that provide insight into the structural, thermodynamic, and mechanistic aspects of the reactions of MT with the most intensive electrophiles, gold(I) complexes, cis-dichlorodiammine Pt(II), and the thiol reagent 5,5′-dithio-bis(2-nitrobenzoate), DTNB.

Reactions of MT with gold(I) anti-arthritic drugs or their metabolites to produce aurothioneins occur *in vivo* and may be a mechanism of drug resistance.[58,62] They are also chemically interesting because gold(I) has different coordination requirements than Cd^{2+} or Zn^{2+}. For anionic ligands such as thiolates and cyanide, only linear, two-coordination is observed for gold(I). Three- and four-coordination is important for neutral ligands such as phosphines and arsines, but are not observed even with a large excess of cyanide or thiolate. Thus, it was not clear initially whether gold(I) could enter the well-defined four-coordinate thiolate sites (MS_4) that accommodate zinc and cadmium ions, or whether the protein structure must be adapted to the preferred geometry of the gold(I) ion.

Aurothioneins were prepared using either sub-stoichiometric quantities or large excesses of the oligomeric drug gold sodium

thiomalate ($[AuSTm]_n$).[63] In the first case, the thiomalate ligands are displaced as the gold replaces Zn + Cd in a ratio of 0.69 ± 0.03 (Zn + Cd)/Au bound. EXAFS/XANES results showed that the gold is coordinated to two sulfur ligands at a distance of 229 pm. These results are consistent with chelation by two MT sulfur ligands and inconsistent with isomorphous substitution of gold(I) into the tetrahedral binding sites of the α and β clusters of MT.[63]

When excess AuSTm is employed in reactions with metallothionein, equal amounts of Au and thiomalate bind to the protein. The ratio of metal displaced to gold bound is 0.34 ± 0.01 and gold is coordinated to two sulfur ligands at a distance of 230 pm. As a result, the effective hydrodynamic radius of the aurothionein is greater than for native MT. Such findings suggest monodentate coordination of individual AuSTm moieties at each of the twenty cysteine residues of an unfolded MT chain.[63] The two binding modes described here are also discussed and illustrated in another Comment.[64] No evidence has been reported to date for isomorphous substitution or for formation of a 1:1 gold(I) thiolate structure, in which MT cysteines bridge between adjacent gold(I) ions.

The new gold therapeutic agent, auranofin ($Et_3PAuSAtg$), is a monomeric complex with triethylphosphine and 2,3,4,6-tetra-O-acetyl-β-1-D-thioglucose ligands, which have greater affinity for gold than thiomalate. As a consequence, $Et_3PAuSAtg$ does not displace Zn or Cd from Zn,Cd-MT and gold does not bind to MT.[65,66] In contrast, the more nucleophilic thiolate groups of metal-free metallothionein do react with auranofin. Ecker *et al.* observed that during the reaction, Et_3P is displaced from most (>80%) of the gold which associates with metallothionein.[65]

Et_3PAuCl, the chloride analogue of auranofin, is more reactive toward MT. It displaces Zn^{+2} from Zn,Cd-Mt. Although the structure of the resulting aurothionein has not been studied spectroscopically, the stoichiometry of metal displaced to gold bound, 0.50, suggests that gold binds both as Et_3PAu^{1+} at individual cysteines and also as Au(I) chelated by two protein cysteines.

The preferential displacement of Zn is not surprising, although it is not known whether it is of kinetic or thermodynamic origin.[63,64,67] As discussed above, MT binds Zn^{+2} less tightly than it binds Cd^{+2}. Likewise, in some ligand exchange reactions, Zn^{+} is extracted more rapidly than Cd^{+2}.

The timescale for the reaction of AuSTm with MT permits con-

ventional spectrophotometric analysis, but the UV-visible absorption bands of various gold(I)-thiolate species overlap and are generally nondescript. Therefore, we developed a novel method to follow the reaction using the metallochromic reagents, PAR (Pyridylazoresorcinol) and ZI (Zincon) to monitor the rate of release of Zn or Cd from MT.[47]

AuSTm and metallothioneins with various metal compositions react with rapid, biphasic kinetics. The rates are independent of the AuSTm concentration over three orders of magnitude.

$$\text{rate} = k_{\text{fast}}[\text{MT}] + k_{\text{slow}}[\text{MT}] \tag{9}$$

The biphasic character of the kinetics is reminiscent of some of the ligand substitution reactions and may represent differential reactivity of the two protein domains.

The lack of gold dependence can be explained by two different mechanisms which have yet to be distinguished: rate limiting dissociative processes localized in the protein, such as a metal-thiolate bond dissociation which frees a thiolate ligand for reaction with the gold drug (reaction 10 and Fig. 3, k_6), or a rapid preequilibrium of AuSTm and MT, which stoichiometrically form a reactive adduct that slowly breaks down to products (reaction 11).

$$-\text{M}-\text{S}- \underset{k-1}{\overset{k_1}{\rightleftarrows}} -\text{M S}- \xrightarrow[\text{fast}]{\text{AuSTm}} -\text{SAuSTm} + \text{M} \tag{10}$$

$$-\text{M}-\text{S}- + \text{AuSTm} \overset{K}{\rightleftarrows} -\text{M}(\text{AuSTm})-\text{S}- \xrightarrow{k} -\text{SAuSTm} + \text{M} \tag{11}$$

An intriguing feature of the AuSTm reactions is their lack of kinetic dependence on the metal composition of MT. For Zn_7-MT and Cd_7-MT, the rate constants are experimentally equal (Table (IV). The reactions of AuSTm with four additional mixed-metal MTs, including several copper species, also display rate constants falling within the limits $k_f = 2.7 \pm 1.2 \times 10^{-2}$ sec^{-1} and $k_s = 6.9 \pm 0.9 \times 10^{-4}$. This result makes it difficult to argue that

TABLE IV

Rate constants for metallothionein-electrophile reactions

	Rate Constants	
	Fast Step	Slow Step
AuSTm + Zn_7-MT[47]	2.4×10^{-2} sec^{-1}	7.3×10^{-4} sec^{-1}
AuSTm + Cd_7-MT	2.2×10^{-2} sec^{-1}	7.3×10^{-4} sec^{-1}
DTNB[a] + Zn_7-MT[72]	0.30 [DTNB] $M^{-1}sec^{-1}$ + 2×10^{-3} sec^{-1}	0.048 [DTNB] $M^{-1}sec^{-1}$ + 1.4×10^{-4} sec^{-1}
DTNB + Cd_7-MT	0.28 [DTNB] $M^{-1}sec^{-1}$ + 2.4×10^{-3} sec^{-1}	0.028 [DTNB] $M^{-1}sec^{-1}$ + 0.75×10^{-4} sec^{-1}

[a]DTNB—5,5′-dithio-bis(2-nitrobenzoate).

the kinetics are determined simply be a dissociative process involving the metal-thiolate clusters (reaction 10), since at least the thermodynamic and some of the ligand substitution properties of Cd- and Zn-clusters are distinctly different.

The anti-tumor agent cis-Platin or cis-DDP ($(H_3N)_2PtCl_2$) binds to MT in tumor cells.[60] In model chemical reactions, excess cis-Pt displaces Zn^{+2} from purified Zn_7-MT as between 7 and 10 Pt atoms bind per mole of protein.[68] At sub-stoichiometric ratios of Pt to MT, Zn,Pt-MTs are formed. The two limiting stoichiometries of metal chelation by MT are compatable with the binding of 2–4 Pt atoms per thiolate. In another biologically interesting reaction of cis-DDP with DNA, bifunctional adducts form, which retain the ammonia ligands.[69] A different course of reaction occurs with MT.

Examination of an all-Pt metallothionein by EXAFS spectroscopy indicates that four sulfur or chlorine atoms are coordinated to Pt, three at 226 pm and one at 233 pm.[70] Although retention of a chloride ligand seems unlikely, EXAFS cannot distinguish between Cl and S binding. It is clear that nitrogen does not remain bound to Pt and that thiolate ligands probably occupy all four coordination sites. Confirmation of this result comes from experiments using an analogue of cis-DDP in which radiolabelled ethylenediamine (en) replaces the ammonia ligands.[69] After reaction less than 2% of the Pt bound to the thionein retains ethylenediamine. Since en is less easily displaced from Pt(II) than ammonia due to the chelate effect, it is clear that the ammonias of cis-Pt will also be displaced under similar conditions. Apparently, sulfhydryl groups of MT replace not only the chlorides of cis-DDP but the ammonia ligands as well. Coupled with the results on the stoichiometry of binding of Pt to metallothionein, it is evident that platinum binds in a cluster arrangement involving bridging thiolate ligands. However, considering the strong preference of Pt(II) for square planar complexation, the product is not expected to be isomorphous with the Zn- or Cd-protein.

Kinetics studies have resolved two discrete steps in the generation of platinothionein (Table V).[69] Examining both the formation of Pt-thiolate bonds (284 nm) and the displacement of Zn^{+2} (metallochromic reagent, Zincon), the appearance of Pt-S chromophore occurs in a slow reaction that depends on the Pt concentration (k_s). This is preceded by a Pt-independent lag phase (k_f), in

TABLE V

Rate constants for reactions of Zn_7-MT with $(NH_3)_2PtCl_2$[a]

$[Cl^-]$/mM	$[PO_4^{3-}]_{total}$/mM	[PT]/μM	Method	Observed Rate Constants (sec^{-1})
4	5	50–5000	Pt-S[b]	lag, $k_f = 2 \times 10^{-3}$; $k_s = 5\text{–}40 \times 10^{-5}$
4	5	5–1000	ZI[c]	$k_f = 3 \times 10^{-3}$; $k_s = 2\text{–}13 \times 10^{-5}$
1–600	0	100	Pt-S	$k_s = 6 \pm 2 \times 10^5$ (Cl^- independent)

[a]All reactions were carried out at pH 7.4 and 25° with [Zn_7-MT] = 20 μM.
[b]Pt-S absorbance at 284 nm.
[c]Zincon absorbance at 620 nm.

which there is little change in 284 nm absorbance. Unlike the DNA binding reaction of cis-Pt, the MT reaction is not chloride ion dependent. This indicates that the aquo and chloro complexes react similarly and that the reaction is not rate limited by chloride ion hydrolysis of cis-DDP.

The displacement of Zn is also biphasic. A fast phase, involving the release of one zinc from MT, is independent of the Pt concentration (k_f) and corresponds to the lag phase described above. The slow component (k_s) has the same Pt concentration dependence as Pt-S bond formation, indicating that most of the zinc is displaced in concert with Pt binding. Interestingly, the lag phase (k_f) has a rate constant similar to one identified for ligand substitution reactions and, thus, may relate to the same dissociative events on the protein (Table II and Fig. 3A). The demonstration of Pt-dependent rates for most of the reaction (k_s) together with the chloride ion independence of the substitution of Pt for Zn point strongly to the facile, direct reactivity of Pt with the sulfhydryls of metallothionein.

Metallothionein also interacts with organic electrophiles. DTNB is a disulfide reagent used to assay protein thiols. It undergoes thiol-disulfide interchange rapidly with free thiols and slowly with the metal coordinated thiols of MT.[71] The kinetics of reaction with MT are biphasic with fast (f) and slow (s) steps, both yielding product and displaying first and second order contributions (Table IV):

$$\text{rate} = \{k_{1,f} + k_{2,f}[\text{DTNB}]\}[\text{MT}] + \{k_{1,s} + k_{2,s}[\text{DTNB}]\}[\text{MT}] \quad (12)$$

As shown above in Fig. 3A, the first and second order terms can be explained in terms of dissociative and associative components for the reaction (k_1, and k_2, k_4 or k_1, k_3, k_4). DTNB and some of the ligands in Table II display similar first order rate constants with MT, which we presently interpret as a dissociative process within the metal-thiolate clusters, common to each reaction pathway. Interestingly, none of these reactions occurs at rates approaching the exchange of metals between cluster sites, which must also require metal-thiolate bond cleavages and reformation.

The rate expression shown in Eq. (12) like that involving AuSTm is not significantly affected by the metal content of the MT (Table IV). Thus, the reactions of DTNB and AuSTm with MT are similar in the following aspects: (1) they are both biphasic; (2) they lack any dependence on the MT metal content; and (3) they are slower than the reactions of free thiols with the same reagents. Nevertheless, the two reactions involve different first order processes as seen by comparing the DTNB-independent components of their rate constants.

N-Ethylmaleimide (NEM) is another chromophoric electrophile which is readily attacked by thiol compounds. It reacts directly with purified Zn_7-MT, converting the cysteines stoichiometrically into thioether derivatives and releasing Zn^{2+} from the protein.[72] Together with the DTNB reaction, this example indicates that metal-coordinated sulfhydryl groups in metallothionein retain substantial nucleophilic reactivity toward organic electrophiles.

In summary, a diversity of kinetic pathways exists for reaction of various electrophilic compounds with metallothionein. This may not be surprising. The density of thiol groups in the protein, which constitute seven non-equivalent metal-binding sites and the fact that the sulfhydryl residues are not buried within a much larger protein superstructure make metallothionein a widely reactive protein with non-physiological molecules of pharmacological and toxicological interest.

RELATIONSHIPS BETWEEN THE CHEMISTRY OF METALLOTHIONEIN AND ITS CELLULAR PROPERTIES

The previous sections argue that metallothionein has a unique metal–ligand cluster structure, which is remarkably reactive in a number of different types of reactions. It does not share the general kinetic unreactivity of some other zinc metalloproteins which have been studied. The rich chemistry of the protein, therefore, indicates that its inorganic reactivity might be more important for its biological functions than previously thought. To conclude this comment, we will examine some of results from biological systems which support this contention.

The clearest example involves the formation of mixed metal,

Cd_m,Zn_{7-m}-MT. The complex ^{113}Cd NMR spectrum of ^{113}Cd,Zn-metallothionein, isolated from the liver of rabbits injected with $^{113}CdCl_2$, can only be reproduced *in vitro* by the reaction of $^{113}Cd_7$-MT with Zn_7-MT (reaction 2). In mammalian liver, the time-course of formation of mixed-metal MT occurs as follows: Cd^{2+} binds to any basal metallothionein in the cells, interacts non-specifically with other sites in cells, and induces MT messenger-RNA synthesis to initiate new apo-metallothionein synthesis (Fig. 1 and Refs. 73 and 74). As apo-MT or Zn_7-MT is made, it rapidly sequesters Cd from other parts of the cell to produce Cd_7-MT[75,76] Here, the large apparent stability constant of MT for Cd is crucial because, for example, mitochondria have binding sites for Cd which compete successfully with Cd-complexes having log apparent stability constants of 11–14.[77] Also important are its facile metal exchange properties, which allow Cd rapidly to displace Zn from the protein and which are a distinctive feature of metallothionein in comparison with other proteins (Table I).

Metallothionein synthesis invariably continues on beyond what is necessary to chelate cellular Cd.[24] One infers that Zn_7-MT must form, for, thereafter, Cd_m,Zn_{7-m}-MT begins to appear according to reaction (1). Once the Cd/Zn ratio has stabilized, it remains constant for long periods during which both Cd and Zn are repeatedly lost from and rebound to MT as the protein maintains a steady state cycle of biodegradation (Fig. 1C) and synthesis (A).

What purpose, if any, is served by the inclusion of Zn in the metallothionein product if the only function of the newly synthesized protein is to sequester and, thus, detoxify cellular Cd? We take the view that basal Zn-metallothionein contributes to essential metal metabolism in cells. Thus, if only enough apo-MT is synthesized to bind Cd in the response described above, cells would be essentially devoid of necessary Zn-metallothionein. Therefore, Cd,Zn-metallothionein is made in order to have at least a population of relatively homogeneous 3-metal clusters of zinc available for participation in aspects of Zn metabolism even when the protein has a large ratio of Cd to Zn.[35]

Once metal-containing metallothioneins have been made, it has been thought that their biodegradation rate constants reflect the properties of rate-limiting hydrolysis of the protein structure by intracellular proteases.[12,78] This view is based on the parallel loss

of radiolabel and Zn from zinc-induced Zn_7-MT in liver and on the assumption that the metals bound to metallothionein are thermodynamically or kinetically inert. Interestingly, the rate constant for biodegradation of induced Zn-metallothionein in liver is 3.5–4.5 times that for Cd,Zn-metallothionein.[11,12] A first hypothesis to explain this difference is that the cadmium and zinc proteins have some significant structural differences leading to differential reactivities with cellular proteolytic enzymes. This idea has its cellular basis in the finding that when the conformation of metallothionein is dramatically altered, as when $(Au(I)\text{-thiomalate})_{20}$-MT is formed in place of Zn_7-MT (see below), its biodegradation rate constant is increased by almost an order of magnitude.[79] Nevertheless, there is no evidence to believe that the Zn- and Cd,Zn-proteins display important conformational differences which could explain their disparate biodegradation rate constants.

An alternative explanation for the parallel rates for Zn loss and protein biodegradation of hepatic zinc metallothionein is that there is an initial, rate-determining event involving the metal clusters followed by rapid hydrolysis of the protein.[78] One obvious possibility in liver is that since much protein biodegradation occurs in lysosomes, where the pH approaches 5, the pH-dependent equilibrium for metal binding favors the dissociation of Zn_7-MT.[20,29] Thus, metal ion dissociation might precede protein turnover. In fact, one study does suggest that at pH 5.0, *in vitro* proteolytic cleavage of Zn_7-MT proceeds more rapidly at lower pH than the reaction with Cd,Zn-metallothionein and that the enzymatic hydrolysis of apo-MT is even faster.[78]

Another hypothesis is that intracellular ligand substitution processes are rate determining for both proteins, followed by rapid degradation of metal-free MT.[80] Since the Zn_7-MT is more accessible thermodynamically and possibly kinetically to competing ligands than Cd_7-MT, it might be expected to react to a greater extent with cellular ligands than the cadmium protein, thus making apo-protein available more quickly for non-rate-limiting biodegradation. Indeed, Zn_7-MT displays a broad pattern of reactivity with small, competing ligands that is not shared by other Zn-metalloproteins (Tables II and III). ^{113}Cd NMR studies showing rapid intersite exchange of metals, particularly in the 3-metal cluster, and providing evidence that some of this activity represents inter-

molecular exchange, further underscores the potential for metallothionein to participate in metal–ligand chemistry in cells.[37]

That Zn-metallothionein may be undergoing ligand substitution reactions in cells is supported by recent studies in Ehrlich tumor cells in animals and culture.[81,82] An expansion of Fig. 1 to include such chemistry (k_6) helps rationalize the results (Fig. 4). It has been demonstrated that under zinc deficient conditions in the external medium, zinc is rapidly lost from the Zn-MT pool on a per cell basis while other soluble Zn-metalloproteins are unaffected within error over a several hour time period. A similar phenomenon is seen also in kidney.[23] These findings differentiate Zn-metallothionein from the rest of the macromolecular pool of Zn. Whatever the mechanism, Zn-metallothionein is the only kinetically labile pool of zinc which can be detected at present in mammalian cells.

When the biodegradation rate for Zn-MT was examined using ^{35}S-cysteine labeled metallothionein in the above experiment, it was observed that the reaction of Zn is several times faster (half-life, 1 hr) than protein hydrolysis (k_3, halflife, 4–10 hr), indicating clearly that biodegradation can not be responsible for the redistribution of MT-bound Zn.[82] Neither can slow dissociation of Zn from MT (k_{-2}) followed by reassociation with apo-Zn metalloproteins (L), for according to the study of the kinetics of reaction of Zn_7-MT with small ligands, k_{-2} is less that 10^{-5} sec^{-1} (Table II, PAR reaction). It has, therefore, been inferred that ligand substitution processes are being observed indirectly in this experiment.

What function do such reactions serve in cells? Possibly to regulate the distribution of Zn to cellular Zn-proteins (k_6 in Fig. 4). One can appreciate the need for some mechanism to control biological metal speciation by simply asking how in the midst of a plethora of potential cellular ligands are the various essential transition metals directed chemically to bind only to particular sites (for example, k_4, in which Cu as well as Zn may react with L).

One experiment which demonstrates the potential for Zn_7-MT to participate in the constitution of apo-Zn proteins in cells involves the addition of apo-carbonic anhydrase to Ehrlich cell supernatant, which contains Zn-metallothionein, other zinc proteins, and few if any small Zn complexes.[81] Even when the apo-protein is present in several-fold excess over Zn-metallothionein, its extent of con-

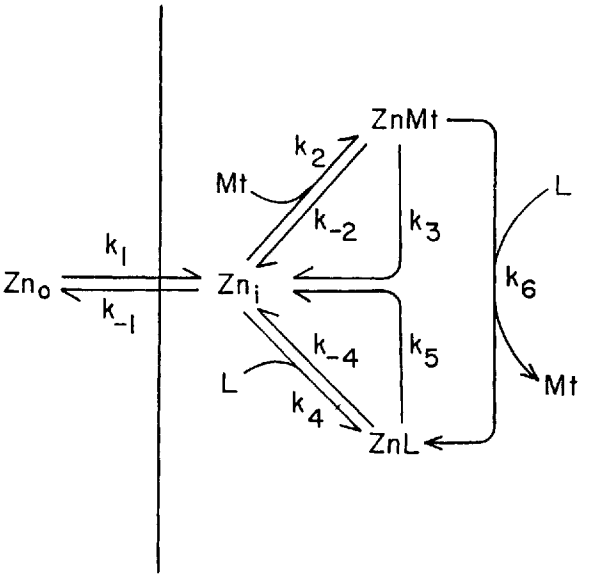

FIGURE 4 Metabolic pathways of zinc in Ehrlich cells. $k_{1,-1}$ represent uptake and efflux of Zn from cell; $k_{2,-2}$, formation and full dissociation of Zn_7-MT; $k_{3,5}$, biodegradative hydrolysis of Zn_7-MT and Zn-L, the combined pool of other Zn-proteins; $k_{4,-4}$, formation and full dissociation of the aggregate of Zn-proteins other than MT; k_6, ligand substitution reaction of L with Zn_7-MT.

version to Zn-carbonic anhydrase is strictly limited by the concentration of Zn associated with MT. Other proteins or pools of Zn do not provide metal for the formation of Zn-carbonic anhydrase. While it is too early to postulate a pervasive role for metallothionein in the cellular deposition of Zn in apo-Zn proteins, its known properties suggest that it may play a role in the specific constitution of certain apo-proteins with zinc. It does possess a moderately large equilibrium constant for Zn, which is in the range of those for apo-carboxypeptidase, apo-carbonic anhydrase, and apo-superoxide dismutase.[38,50,83] In addition, it displays facile reactivity of Zn_7-MT with apo-carbonic anhydrase as well as with numerous small chelating agents. Finally, it exhibits apparent lability in transferring zinc to other sites in cells.

Although the primary focus of attention in metallothionein research has been on the binding of essential and toxic metals, me-

tallothionein is also a prominent site of reaction of therapeutic gold and platinum complexes.[58,62,84] Neither Au nor Pt complexes cause the accumulation of metallothionein. Cis-platin appears only to bind to thiol groups of preexistent protein and not to induce its synthesis. In contrast, gold drugs induce m-RNA synthesis in cell culture leading to enhanced protein synthesis.[79] However, this is offset by the accelerated biodegradation of aurothionein (halflife = 0.75 hr, compared to 10 and 24 hr for Zn-MT and Cd-MT, respectively). The perturbations of the MT structure required to accommodate the linear geometry of the gold described above may make this species more susceptible to attack by cellular proteolytic enzymes than the Zn- or Cd-protein.

Understanding of the chemical properties of the interactions of Au and Pt complexes with metallothionein helps to explain some aspects of their biological behavior. Thus, the observation that cultured cells exposed to auranofin form aurothioneins, even though there is no direct reaction *in vitro* between drug and Zn-metallothionein, can be rationalized by the differences in the reactivity of AuSTm, Et_3PAuCl and $Et_3PAuSAtg$ with the protein.[67] Apparently, biological ligand exchange reactions lead to loss of AtgSH from auranofin before or during transport into cells, producing a structure such as Et_3PAuCl that is reactive with metallothionein.

The rate of gold binding to MT in chemical experiments is much greater than the rate at which aurothioneins form *in vivo* after administering gold to test animals, indicating that other processes, possibly membrane transport phenomena, are the rate limiting step in the appearance of intracellular gold-metallothionein species.

The findings in our Pt-drug studies that cis-DDP reacts with Zn_7-MT with kinetics that are independent of chloride concentration and faster than the chloride hydrolysis constants for the complex also have implications for the mechanistic understanding of the reactivity of cis-DDP in biological systems. As reviewed elsewhere, the qualitative picture has developed from chemical studies that the drug is relatively inert in plasma where $[Cl^{-1}]$ is 0.1 M, because it exists in the unreactive, dichloro form, but becomes activated in cells where $[Cl^{-1}]$ is 0.004 M as the monaquo and diaquo species appear.[85] The aquation reactions which generate these reactive forms are quite slow. Yet, according to biological studies, cis-DDP rapidly (1–2 hrs) binds to molecules in blood as well as to com-

ponents of cells and inhibits DNA synthesis in this same time frame.[85] The recognition that metallothionein reacts with the Pt-drug with Pt-dependent kinetics that are not rate limited by chloride dissociation (Table V) suggests a view of its biological reactivity that is consistent with other aspects of its chemistry, such as the direct reaction of the dichloro-complex with strong nucleophiles including sulfhydryl groups as well as with the biological behavior of the complex (Fig. 3).

The importance of the reactions of gold or platinum drugs with MT is uncertain. One idea is that because of these reactions, the concentrations of active forms of the drugs are reduced, thereby lowering their efficacy. That is, MT protects cells from these agents. Thus, cells that overproduce MT have unusual resistance to the cytotoxic effects of platinum.[59] Indeed, a recent paper shows that some cell types made resistant to cis-dichlorodiammine Pt(II) or even to organic electrophiles such as L-phenylalanine nitrogen mustard display substantially elevated levels of metallothionein.[86] If MT acts in this way, then a variety of commonly used rodent tumors, particularly solid tumors, may have significant protection against the platinum drug, for generally they contain a substantial amount of metallothionein not seen in normal tissues even before these tumors become resistant to further treatment.[87]

That organic, electrophilic antitumor agents may also preferentially react with metallothionein is consistent with our studies showing that even the metal-bound thiolate ligands of MT retain significant nucleophilic reactivity with model electrophiles such as DTNB (Table IV) and NEM. If this is a quantitatively important reaction in cells, which also have large amounts of the tripeptide thiol, glutathione, then one asks whether the presence of high levels of MT in some tissues may also play a role in the metabolism of a variety of non-metallic electrophilic species, including organic compounds and radical and oxy-radical species.

While it is attractive to view such reactions as contributing to the detoxification of foreign reactive entities, to the extent that they reduce the functionally important level of metallothionein in the cell, these reactions might also contribute to toxicity.[88] Accordingly, the development of resistance to an agent through selection of cells which have elevated production of metallothionein might stem either from the non-specific affinity of MT for the

reagent or from a specific need of cells for functional metallothionein. The latter is seen, for example, in the response of tumor cells to the antifolate, methotrexate, in which the site of toxic action of the compound, dihydrofolate reductase, is overproduced to overcome the deleterious effects of the drug.[89]

Acknowledgments

The authors acknowledge NIH grants ES-04026, ES-04036, and ES-04061 for continued support for their research on metallothionein. Our work has also benefited from NMR instrumentation funded by the Division of Research Resources of the NIH (RR 04095) and from the Biological and Chemical Instrumentation Programs of the NSF (DIR-8719808). DHP recalls with gratitude and pleasure some of his origins in the area of bioinorganic chemistry in the laboratory of Fred Basolo, editor of this journal.

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