^{111}Cd NMR Studies of the Domain Specificity of Ag^+ and Cu^+ Binding to Metallothionein†

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ABSTRACT: Metal displacement reactions of Cd_7MT with Ag^+ or Cu^+ and interprotein metal exchange reactions between Cd_7MT and Ag_{12}MT or Cu_{12}MT were studied by ^{111}Cd NMR. Titration of $^{111}Cd_7$MT with Ag^+ indicates that Ag^+ binds preferentially to the β-domain of the protein to form the metal hybrid species, $(Cd_4)^{\alpha}(Ag_6)^{\beta}$MT. Once the β-domain is filled, additional Ag^+ ions displace Cd^{2+} from the α-domain to form $(Ag_6)^{\alpha}(Ag_6)^{\beta}$MT. The metal displacement reaction is cooperative and the two domains react independently of one another. The $(Cd_4)^{\alpha}(Ag_6)^{\beta}$MT hybrid protein is also formed as the major product of direct interprotein metal exchange between Cd_7MT and Ag_{12}MT. Cu^+ reacts with Cd_7MT in a manner similar to Ag^+, with addition of 6 equiv of Cu^+ leading to preferential formation of $(Cd_4)^{\alpha}(Cu_6)^{\beta}$-MT, and 12 equiv of Cu^+ to formation of $(Cu_6)^{\alpha}(Cu_6)^{\beta}$MT. However, unlike Ag^+, Cu^+ appears to produce intermediate species that may contain mixed-metal clusters. Interprotein metal exchange between Cu_{12}-MT and Cd_7MT leads to the net transfer of Cd^{2+} into the α-domain and Cu^+ into the β-domain. The differential affinities of the two domains for monovalent and divalent metal ions plus the availability of facile pathways for metal exchange may be features that enable MT to function simultaneously in the metabolism of different metal ions.

Mammalian metallothioneins (MTs)[1] [see Kägi (1993) for review] comprise a family of small, cysteine-rich proteins that *in vivo* bind the essential metal ions Zn^{2+} and Cu^+ and the toxic metal ion Cd^{2+}. In animals not exposed to elevated levels of Cd or Cu, the most abundant, and often sole, metallic constituent of MT is Zn, whereas in fetal and neonatal tissues MT contains relatively high proportions of Cu in addition to Zn [see Cherian and Chan (1993) for review]. Exposure to Cd or Cu leads to production not of homogeneous all-Cd or all-Cu MTs, but to mixed-metal Zn,-Cd- or Zn,Cu-MTs. Proposed functions of the protein include its participation in regulation of intracellular zinc and copper concentration and bioavailability, as well as cadmium detoxification (Webb, 1987; Templeton & Cherian, 1991).

The metals in MT are coordinated exclusively by cysteine residues located in conserved positions in the sequence that are organized to favor formation of two distinct metal thiolate clusters in separate structural domains. Zn^{2+}, Cd^{2+}, and other divalent metal ions form tetrahedrally-coordinated Me_3Cys_9 and Me_4Cys_{11} clusters in the N-terminal β-domain and C-terminal α-domain, respectively (Otvos & Armitage, 1980; Winge & Miklossy, 1982; Robbins et al., 1991). Monovalent Cu^+ forms substantially different complexes with MT, the structures of which are not yet well characterized. Several studies have sought to define the stoichiometry and structures of the metal clusters formed by copper binding to MT and have produced varying results. Factors contributing to this variability are the oxidative susceptibility of bound Cu^+, its spectroscopically silent nature, and the stability of "over-metalated" forms of the protein that may not exist *in vivo*. Nevertheless, there is general agreement that Cu^+ binds much more tightly to MT than Cd^{2+} or Zn^{2+} and interacts with the thiolate ligands in a different fashion, forming complexes with a thiolate/metal ratio closer to 2 (Geller & Winge, 1982; Rupp et al., 1979) than the usual 3 observed for divalent metal ions. The all-Cu protein has been made *in vitro* by sequential displacement of Zn^{2+} or Cd^{2+} from MT by Cu^+ or by reconstituting apo-MT with Cu^+ (Nielson et al., 1985; Rupp & Weser, 1974; Gasyna et al., 1988; Li & Weser, 1992; Pountney et al., 1994). In most cases, spectroscopic titrations were observed to plateau at around 12 copper equivalents, although stoichiometries ranging from 8 to 20 have been reported (Geller & Winge, 1982; Rupp & Weser, 1974; Nielson et al., 1985; Stillman et al., 1987; Zelazowski et al, 1989; Pountney et al., 1994). Hepatic MT isolated from acutely copper-overloaded individuals was found to contain about 12 equiv of Cu (Hunziker & Sternlieb, 1991). However, a different form of CuMT containing only about 8 equiv of Cu has been reported to be present in hepatic lysosomes of Bedlington terriers (Johnson et al., 1981), which inherit an abnormality of copper metabolism similar to Wilson's disease in humans (Scheinberg & Sternlieb, 1976). Extended X-ray absorption fine structure (EXAFS) data on this protein and *in vitro*-generated Cu_8MT (Pountney et al., 1994) suggested the presence of two adamantane-like Cu_4-clusters analogous to those found in MTs containing divalent metal ions (Freedman et al., 1986). In contrast, the metal complexes of *in vitro*-generated Cu_{12}MTs studied by EXAFS and other spectroscopic and physical methods appear to

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[1] Abbreviations: AAS, atomic absorption spectroscopy; MT, metallothionein; apo-MT, metal-free metallothionein; MT1 and MT2, metallothionein isoforms 1 and 2, respectively; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; ppm, parts per million; Tris, tris(hydroxymethyl)aminomethane; ESR, electron spin resonance spectroscopy.

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consist of two Cu_6Cys_{9-11} clusters with the Cu^+ coordinated both trigonally and digonally by cysteine thiolate ligands (Nielson & Winge, 1984; Nielson et al., 1985; George et al., 1986, 1988; Pickering et al, 1993).

In addition to the different binding affinities and coordination preferences of divalent (Zn^{2+} or Cd^{2+}) and monovalent (Cu^+) ions, the two classes of metal ions appear to interact differentially with the two structural domains of MT. Two studies have found evidence for preferential binding of Cu to the N-terminal β-domain in mixed-metal metallothioneins. The first indicated that the Cu in native calf liver Zn,CuMT exclusively occupies the β-domain in a 3-metal cluster (Briggs & Armitage, 1982). This conclusion was based on the ^{113}Cd NMR spectrum of the protein following selective *in vitro* replacement of the Zn by ^{113}Cd. Only resonances corresponding to the α-domain 4-metal cluster were observed, which had essentially identical chemical shifts to those of rabbit liver MT. In the other study, it was found that when Cu^+ was added to apo-metallothionein from rat liver, the metal bound preferentially and cooperatively to the thiolates of the β-domain based on proteolysis experiments (Nielson & Winge, 1984). Despite agreement that the preferred site of Cu binding is to the β-domain, the studies differed with regard to the Cu stoichiometry of the clusters, the former indicating a Cu_3Cys_9 cluster and the latter a $Cu_{(5-6)}Cys_9$ structure. Another study of native calf liver Zn,-CuMT suggested that the Cu ions are largely complexed in the β-domain but also to a smaller extent in the α-domain (Winge et al., 1986).

Mixed-metal metallothioneins are the most prevalent species found *in vivo*, and their metal compositions vary extensively depending on a variety of physiological factors. Little information is available about how these species form and what influence is exerted on the chemical properties of one metal by the binding of another to the same molecule. In a previous study, we showed using Cd NMR that Zn and Cd are not distributed randomly among the multiple metal binding sites of native Cd,ZnMT but rather form a limited number of mixed-metal clusters with Cd located predominantly in the α-domain and Zn in the β-domain. Moreover, it was demonstrated that these specific mixed-metal species are produced by an intermolecular metal exchange reaction between Zn_7MT and Cd_7MT (Nettesheim et al., 1985). Since copper is invariably found *in vivo* in mixed-metal Zn,CuMTs, it is of interest to determine how Cu interacts with Cd- or Zn-containing MTs, which, if any, mixed-metal clusters are formed, and whether the same species can be produced by intermolecular metal exchange between CuMT and ZnMT. Answers to these questions are of importance in reaching a fuller appreciation of metallothionein's function(s) in metal metabolism.

A direct comparison of the binding properties of Cu^+ and Ag^+ is necessary because of the possibility of employing $^{109}Ag^+$ as an air-stable, NMR-active analog of Cu^+ in future structural studies of mammalian MT, analogous to those conducted successfully on yeast MT (Narula et al., 1991). Evidence for the close similarity of Cu^+ and Ag^+ binding to mammalian MT has previously been obtained by others (Nielson et al., 1985; Winge, 1987; Stillman et al., 1988; Zelazowski et al., 1989; Zelazowski & Stillman, 1992). In this study, we use ^{111}Cd NMR to probe the structures of the mixed-metal MT species produced when monovalent Ag^+ or Cu^+ are titrated into Cd_7MT to replace the divalent Cd^{2+}, to characterize the products of reaction of CdMT with AgMT or CuMT, and to address the question of how monovalent Ag^+ and Cu^+ are partitioned among the two structural domains of MT when occupancy is shared with divalent metal ions.

MATERIALS AND METHODS

Isolation and Purification of MT. ZnMT was isolated from the livers of 3−7 pound white New Zealand rabbits injected with 0.15 M $ZnSO_4$ solution for six consecutive days in the amount of 20 mg of Zn/lb/day. 6 h after the final injection, the rabbits were sacrificed and the livers excised and stored at −80 °C. One rabbit liver was used for each preparation. After thawing overnight at 4 °C, the tissue was cut into small pieces and homogenized in a blender with 100−200 mL of 0.25 M sucrose and 5 mM β-mercaptoethanol in 5 mM Tris-HCl, pH 8.6. The homogenate was ultracentrifuged at 143 000*g* for 1 h at 5 °C. The supernatant was then applied to a 10 × 100 cm column of Sephadex G-75 at 4 °C and eluted with 5 mM Tris-HCl, pH 8.6, containing 0.02% NaN_3. The ZnMT fractions were identified by their zinc content as assessed by AAS and pooled. Further separation of the MT1 and MT2 isoforms was achieved on a 2.4 × 35 cm column of DEAE cellulose (Whatman BioSystem Ltd.). The protein was eluted using a 2 L linear gradient from 5 to 300 mM Tris-HCl, pH 8.6, at room temperature. The fractions corresponding to ZnMT1 and ZnMT2 were pooled separately and then concentrated and desalted by ultrafiltration using an Amicon YM2 membrane under N_2 pressure at 4 °C. Only MT2 was used in this study.

Preparation of ^{111}CdMT. ^{111}CdMT was obtained by direct displacement of the Zn^{2+} from ZnMT by $^{111}Cd^{2+}$ (96% enriched from Isotec, Inc.). The following procedure was used to avoid oligomerization of the protein that can occur during the metal exchange process. An aliquot of a $^{111}CdCl_2$ stock solution (*ca.* 1 M) corresponding to 7 equiv of Cd^{2+} was added to a solution of ZnMT (*ca.* 0.015 mM in 20 mM Tris-HCl, pH 8.6) along with Chelex-100 resin (Bio-Rad Laboratories) (*ca.* 5 g per 50 mL solution) and stirred gently. Chelex-100 functions to remove free Zn^{2+} and excess Cd^{2+}, which cause MT oligomerization at higher protein concentrations. After approximately 30 min, the Chelex was allowed to settle and the concentrations of both Zn and Cd in the supernatant were measured by AAS. The procedure was repeated until the Zn concentration was less than 2% of the Cd concentration. The resulting ^{111}CdMT was separated from the Chelex by filtration and then concentrated and desalted by ultrafiltration.

Metal Solutions. Ag^+ solutions were prepared from $AgNO_3$. Cu^+ solutions were prepared from the stable $[Cu(CH_3CN)_4]ClO_4$ complex synthesized according to the procedure of Hemmerich and Signart (1963). The stock solution of Cu^+ had a concentration of 100 mM in 50% H_2O/50% CH_3CN and contained no detectable Cu^{2+} as indicated by its lack of ESR signal and reactivity with bathocuproine disulfonic acid (Zak, 1958).

Protein Characterization. Cysteine-SH concentrations were measured using 5,5′-dithiobis(nitrobenzoic acid) (DTNB) in 6 M guanidine hydrochloride (Ellman, 1959). Cd, Ag, and Cu concentrations were determined by AAS using a Perkin Elmer-3100 atomic absorption spectrometer. Protein concentrations of Zn_7MT and Cd_7MT were measured spectrophotometrically at 220 nm in 0.01 M HCl using the

extinction coefficient for apo-MT of 47 300 M^{-1} cm^{-1} (Bühler & Kägi, 1979).

Metal Binding Reactions. Metal displacement experiments were carried out by adding aliquots of concentrated Ag^+ or Cu^+ stock solutions to NMR samples (2 mL) containing 1.5 mM $^{111}Cd_7MT$. Prior to metal addition, Chelex-100 (*ca.* 2 g) was added to the protein solution contained in a conical centrifuge tube. After each ^{111}Cd NMR spectrum was run, a 5 μl aliquot was withdrawn and saved for metal composition determination and/or DEAE HPLC analysis [see Li and Otvos (1996)]. Prior to the next metal ion addition, the protein was transferred to the same centrifuge tube containing the original Chelex.

The interprotein metal exchange reactions were conducted by incubating 2 mL of 1.5 mM $^{111}Cd_7MT$ with increasing amounts of $Ag_{12}MT$ or $Cu_{12}MT$. The $Ag_{12}MT$ and Cu_{12}-MT used in these reactions were obtained from the final titration step of the reactions described above of Cd_7MT with Ag^+ or Cu^+ monitored by ^{111}Cd NMR. Prior to their use, the $Ag_{12}MT$ and $Cu_{12}MT$ samples were concentrated about 3-fold by ultrafiltration using a Centricon-3 ultrafilter (Amicon).

All manipulations involving Cu^+ and Cu^+-containing MT were carried out in an anaerobic chamber under 5% H_2/95% N_2 atmosphere.

^{111}Cd NMR Experiments. ^{111}Cd NMR spectra were acquired at 106 MHz on a General Electric GN-500 spectrometer without proton decoupling at 30 °C. The samples were contained in 10 mm tubes containing 10% D_2O for field-frequency lock. An air-sensitive NMR tube (J. Young valve tube from Wilmad) was used in the experiments involving Cu^+ and Cu^+-containing protein. The chemical shift of ^{111}Cd was referenced to external 0.1 M $^{111}Cd(ClO_4)_2$. Typical acquisition parameters were: 60° observation pulse, 335 ms acquisition time, 15 000 Hz spectral width, and 336 ms pulse repetition rate. Each ^{111}Cd NMR spectrum required 4−5 h to obtain.

In the titrations of Cd_7MT with Ag^+ or Cu^+, NMR data collection was typically begun about 1 h after the Ag^+ or Cu^+ additions were made. In the metal exchange reactions of Cd_7MT with $Ag_{12}MT$ or $Cu_{12}MT$, spectral acquisition began 30−60 min after $Ag_{12}MT$ or $Cu_{12}MT$ was added. When repeat spectra of the same protein preparation were obtained, there was no indication of any significant time-dependent spectral changes. DEAE HPLC experiments [described in Li and Otvos (1996)] on protein prepared under these conditions also confirmed that the reactions that were monitored by Cd NMR were at least 95% complete by the time the NMR spectra were collected. Each titration experiment was repeated at least twice.

RESULTS

Reactions of Cd_7MT with Ag^+. The demonstrated order of binding affinities of metals to metallothionein *in vitro* is Cu,Ag > Cd > Zn (Nielson et al., 1985; Rupp & Weser, 1979), which makes it possible for Ag^+ or Cu^+ to stoichiometrically displace Zn^{2+} or Cd^{2+} from MT. Such metal displacement reactions are kinetically facile and are generally complete within a few seconds. Owing to the great sensitivity of Cd chemical shifts to metal coordination environment (Summers, 1988), we undertook the use of Cd NMR to monitor the progressive structural changes that occur upon addition of Ag^+ or Cu^+ to Cd_7MT. The results of a Ag^+

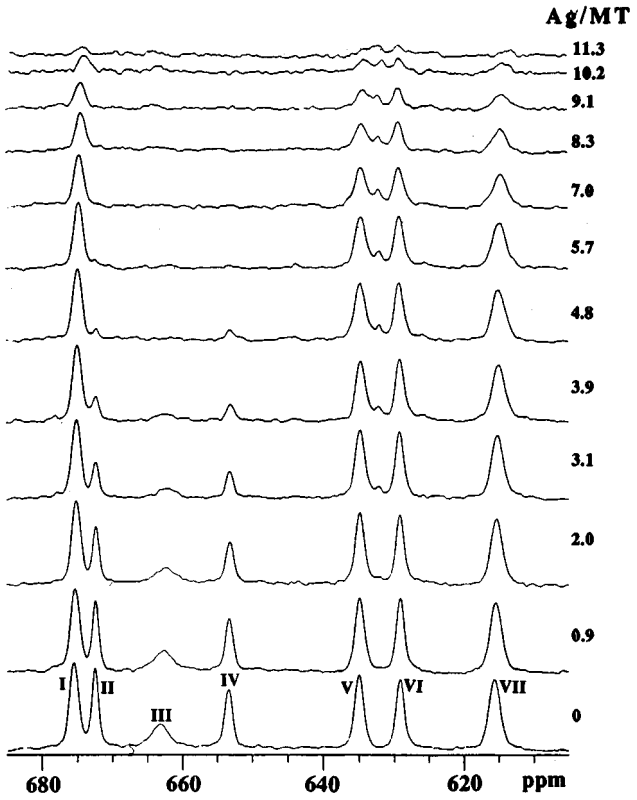


FIGURE 1: ^{111}Cd NMR titration of 1.6 mM $^{111}Cd_7MT$ with Ag^+. 40 000 transients were collected for each spectrum. Peaks labeled I, V, VI, and VII arise from Cd^{2+} in the α-domain 4-metal cluster, and peaks II, III and IV arise from Cd^{2+} in the β-domain 3-metal cluster.

titration of $^{111}Cd_7MT$ are shown in Figure 1. Peaks labeled I, V, VI, and VII are due to the Cd^{2+} ions in the α-domain 4-metal cluster, and peaks II, III, and IV arise from the metals bound to the β-domain 3-metal cluster (Otvos & Armitage, 1980). The spectra demonstrate that as Ag^+ is added early in the titration the intensities of the resonances corresponding to the 3-metal cluster progressively decrease while the 4-metal cluster signals remain unchanged. The peaks corresponding to Cd in the 4-metal cluster begin to decrease after the resonances from the 3-metal cluster completely disappear at a Ag/MT ratio of about 6. These four resonances then progressively lose intensity as more Ag^+ is added until they disappear completely at a Ag/MT ratio of approximately 12. The plot in Figure 2 shows the changes in the summed areas of the signals from each cluster during the metal displacement reaction. The results clearly indicate that intensity from the 4-metal cluster signals remains constant while decreases occur in intensity of the 3-metal cluster signals; only after the 3-metal cluster signals disappear do the 4-metal cluster signals begin to disappear.

Figure 1 also shows that with the exception of a small resonance at 632 ppm, no new peaks were observed during the titration. In addition, the peak intensities corresponding to the Cd^{2+} ions in each domain decrease to the same extent upon each addition of Ag^+. Finally, the chemical shifts and shapes of the peaks corresponding to both the 3- and 4-metal clusters remain unchanged throughout the titration.

The metal content of each titrated sample measured by AAS showed a progressive decrease in Cd concentration and a corresponding increase in Ag concentration (data not shown). Thus, the observed intensity decreases of the Cd NMR signals are due to Cd^{2+} displacement from the protein as Ag^+ binds.

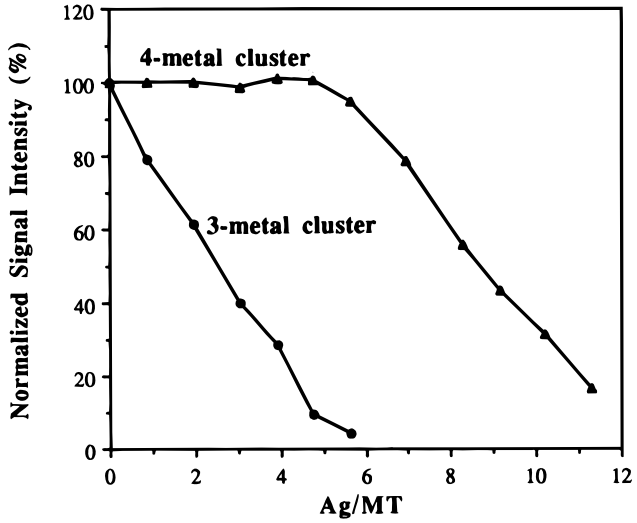


FIGURE 2: Summed ^{111}Cd NMR signal intensities from the four α-domain resonances and three β-domain resonances at different stages of the titration of $^{111}Cd_7MT$ with Ag^+ shown in Figure 1.

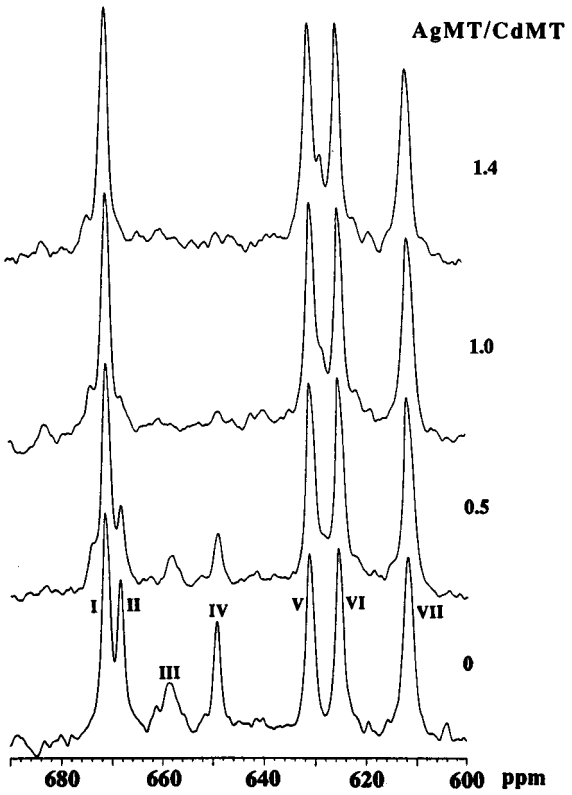


FIGURE 3: Titration of $^{111}Cd_7MT$ with $Ag_{12}MT$ monitored by ^{111}Cd NMR. 1.5 mM Cd_7MT was titrated with aliquots of 7.0 mM $Ag_{12}MT$, and 55 000 transients were collected for each spectrum.

Metal Exchange Reaction between Cd_7MT and $Ag_{12}MT$. The findings that Zn_7MT and Cd_7MT can undergo facile intermolecular metal exchange reactions that partition the two metals differentially between the two clusters (Nettesheim et al., 1985) and that HgMT and CdMT also appear to undergo similar reactions (Johnson & Armitage, 1987) suggest that the same reaction between CuMT and ZnMT or CdMT might be responsible for producing the native Zn,-CuMTs and Cd,CuMTs that are found *in vivo* (Hamer, 1986; Kojima & Kägi, 1978). To begin to investigate this possibility, a titration of $^{111}Cd_7MT$ with $Ag_{12}MT$ was monitored by ^{111}Cd NMR (Figure 3). Qualitatively, the decrease in the intensity of the 3-metal cluster resonances was similar to results that were obtained in the initial stages

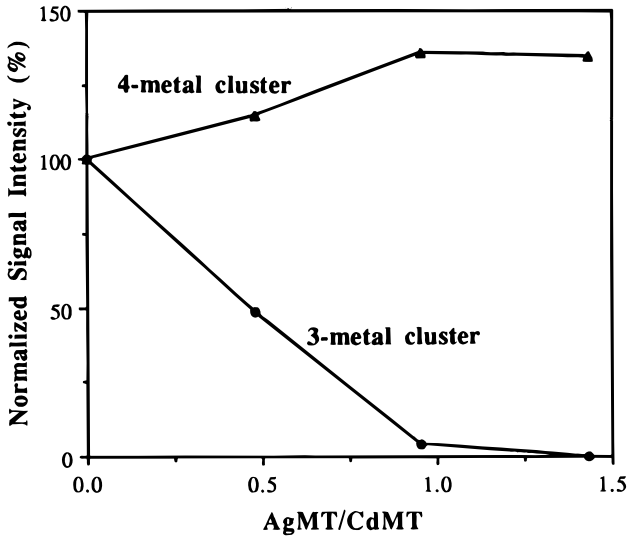


FIGURE 4: ^{111}Cd NMR signal intensity from the α-domain 4-metal cluster and β-domain 3-metal cluster at different stages of the titration of $^{111}Cd_7MT$ with $Ag_{12}MT$ shown in Figure 3.

of the titration of $^{111}Cd_7MT$ with Ag^+ (as shown in Figure 1). That is, the intensities of the resonances corresponding to the 3-metal cluster progressively decrease and their chemical shifts remain unchanged. At the same time, the intensities of the 4-metal cluster resonances increase. These changes in the intensities of the ^{111}Cd resonances from the two clusters are shown graphically in Figure 4. As Ag_{12}-MT is titrated into Cd_7MT, the signal intensity from the α-domain 4-metal cluster increases and that from the β-domain 3-metal cluster decreases until the AgMT to CdMT ratio reaches about 1. Thereafter, the intensities of the 4- and 3-metal cluster signals remain constant as more Ag_{12}-MT is added. These results indicate a net transfer of Ag^+ from α- to β-domains *via* interprotein metal exchange, accompanied by movement of Cd^{2+} from β- to α-domains. Figure 3 also indicates that, as in the titration of Cd_7MT with Ag^+ (Figure 1), the chemical shifts and peak shapes corresponding to both the 3- and 4-metal clusters remain unchanged throughout the titration of Cd_7MT with $Ag_{12}MT$.

Reactions between Cd_7MT and Cu^+ or $Cu_{12}MT$. Although experimentally more demanding owing to the sensitivity of Cu^+ and CuMTs to oxidation, the same two types of experiments described above with Ag^+ were carried out with Cu^+. Strictly anaerobic conditions were used in order to minimize artifacts due to oxidative damage. Figures 5 and 6 show the results of displacing Cd^{2+} from Cd_7MT with Cu^+ and the metal exchange reaction between Cd_7MT and Cu_{12}-MT, respectively. The general patterns of the changes in peak intensities were similar to those obtained with Ag^+ (Figures 1 and 3). That is, the intensity of 3-metal cluster signals decreased early in the titration, followed by a reduction in intensity of the 4-metal cluster signals after the 3-metal cluster signals disappeared.

However, the detailed pattern and the stoichiometry of the Cu^+ displacement reaction were consistently different from what was observed in the Ag^+ titration. The data in Figure 5 indicate that as Cu^+ is initially added, the intensities of the 4-metal cluster signals remain unchanged while those of the 3-metal cluster decrease, and at the same time some small new peaks appear at 621, 617, and 596 ppm (labeled 1, 2, and 3, respectively). At a Cu/MT ratio of about 3, the *original* resonances from the 3-metal cluster completely

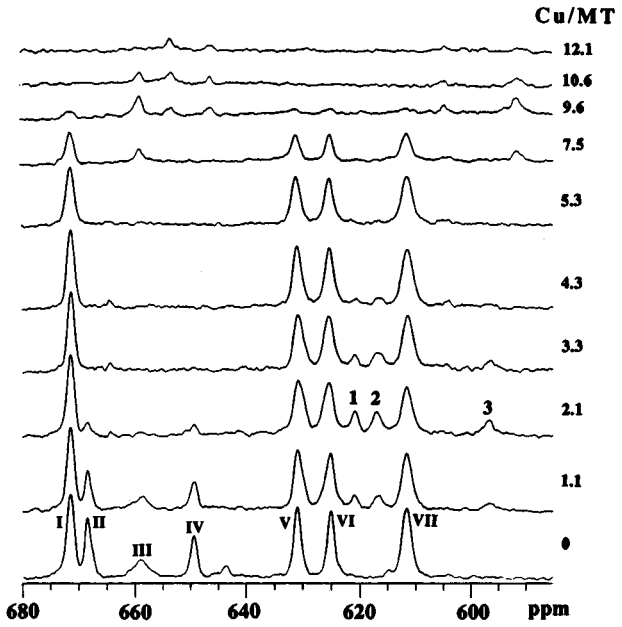


FIGURE 5: ^{111}Cd NMR titration of 1.5 mM $^{111}Cd_7MT$ with Cu^+. 45 000 transients were collected for each spectrum.

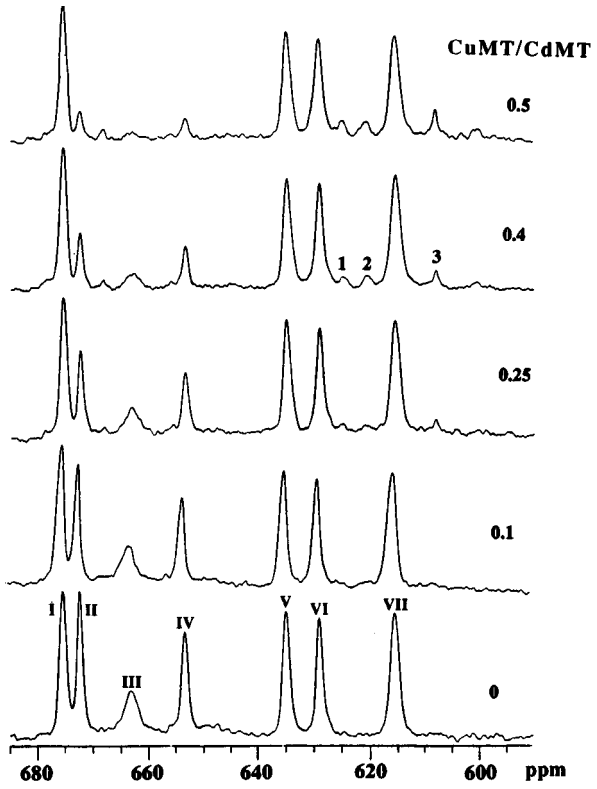


FIGURE 6: Titration of $^{111}Cd_7MT$ with $Cu_{12}MT$ monitored by ^{111}Cd NMR. 1.3 mM $^{111}Cd_7MT$ was titrated with aliquots of 3.2 mM $Cu_{12}MT$, and 55 000 transients were collected for each spectrum.

vanish and the new peaks have their maximum intensities. As shown in Figure 5, essentially the only spectral changes brought about by addition of the next 2−3 equivs of Cu^+ were the disappearance of the new resonances 1, 2, and 3. Further additions of Cu^+ caused a decrease in the intensities of the 4-metal cluster resonances. At a Cu/MT ratio of about 10, the *original* peaks from the 4-metal cluster vanished while at least five other new peaks appeared. Subsequently, as more Cu^+ was added, these new peaks also disappeared at a Cu/MT ratio of about 12.

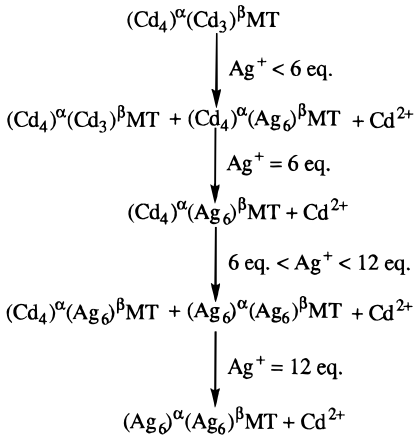
Shown in Figure 6 is evidence that interprotein metal exchange takes place when Cd_7MT and $Cu_{12}MT$ are mixed together, just as was seen in Figure 3 for the reaction of Cd_7MT and $Ag_{12}MT$. What is different is that reduction of the 3-metal cluster signals of Cd_7MT as $Cu_{12}MT$ was added was not accompanied by a corresponding increase in intensity of the 4-metal cluster signals. Instead, just as occurred by addition of Cu^+ ion, several new resonances (labeled 1, 2, and 3) were seen to grow into the spectrum while the 4-metal cluster signals decreased somewhat. These results suggest that, at relatively low CuMT/CdMT ratios, interprotein metal exchange leads to the net transfer of α-domain Cu^+ into β-domains to share occupancy with Cd^{2+} (see below).

DISCUSSION

The metal composition of metallothionein *in vivo* is usually heterogeneous and varies widely depending on physiological circumstances. A key question concerning the postulated functions of MT in detoxification and essential metal metabolism is whether its two structurally distinct metal-thiolate clusters, located in well-separated protein domains, endow it with the ability to play multiple roles. Having two clusters with different coordination properties might allow one to serve as a relatively inert long-term storage site used for detoxification or homeostatic purposes while the other could function as a more dynamic site of metal interaction at which metal transfer reactions might preferentially occur. Our previous Cd NMR studies suggest that there are facile kinetic pathways for direct metal interchange between MT molecules which lead to partitioning of different metals between the two domains. *Via* interprotein metal exchange between CdMT and ZnMT, the Cd in Cd,ZnMTs becomes sequestered almost exclusively in the α-domain 4-metal cluster while Zn predominantly partitions into the β-domain 3-metal cluster (Nettesheim et al., 1985; Otvos et al., 1987). Since the protein formed *in vivo* following exposure to Cd contains the same mixture of Cd,-Zn hybrid MT species generated *in vitro* by interprotein metal exchange, the reaction is believed to have physiological relevance (Nettesheim et al., 1985).

The question of how monovalent metal ions such as Cu^+ and Ag^+ are distributed between the two domains of MT when these metals share occupancy of the protein's binding sites with divalent metal ions such as Zn^{2+} and Cd^{2+} is perhaps even more important. $Cu_{12}MT$ and $Ag_{12}MT$ are known to form metal-thiolate clusters that differ in stoichiometry and structure from those formed by the divalent metal ions in Zn_7MT and Cd_7MT (Geller & Winge, 1982; Rupp et al., 1979). Under many physiological conditions, particularly in neonatal tissue, MT contains predominantly Cu, but also substantial amounts of Zn and sometimes Cd as well (Cherian & Chan, 1993). With protein isolated by standard methods, it is not readily possible to determine in these Zn,Cu-, Cd,Cu-, and Cd,Zn,Cu-MTs whether the Cu, Zn, and Cd are located in the same or different MT molecules. If both monovalent and divalent metals are indeed bound to the same MT molecule, as our results suggest, it is important to know whether they share occupancy of the same cluster, or whether they are partitioned between the two domains so as to form homogeneous single-metal clusters in each domain. The NMR experiments reported here clearly indicate that Ag^+ only forms all-Ag clusters and binds preferentially to the β-domain whereas Cu^+ appears capable of forming at least some mixed-metal cluster species.

Scheme 1: Pathway of the Metal Displacement Reaction of Cd_7MT with Ag^+



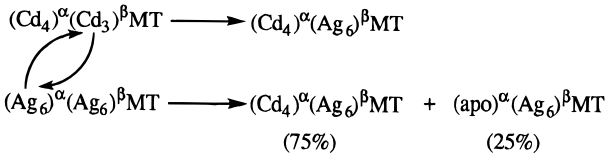
Titration of Cd_7MT with Ag^+ produces remarkably simple Cd NMR spectra (Figure 1) that are subject to straightforward interpretation. Addition of the first 6 equiv of Ag^+ leads to progressive elimination of the three signals arising from the Cd_3Cys_9 β-domain cluster without any perturbation to the four signals from the Cd_4Cys_{11} cluster in the α-domain. Additional Ag^+ up to 12 equiv produces a progressive elimination of the α-domain 4-metal cluster Cd signals. Since the intensities of the peaks arising from each Cd^{2+} in a particular cluster decrease to the same extent upon each addition of Ag^+, and the chemical shifts of the original peaks corresponding to the 3- and 4-metal clusters remain unchanged throughout the titration, it can be concluded that degradation of the Cd clusters in both domains occurs in a cooperative, all-or-nothing fashion. Furthermore, the metal composition and structure of one domain do not appear to have any influence on the metal cluster environment of the other domain.

The results of the metal displacement reaction of Cd_7MT with Ag^+ can be summarized in Scheme 1. This scheme shows that in the first half of the titration increasing amounts of a single mixed-metal $(Cd_4)^{\alpha}(Ag_6)^{\beta}MT$ complex are produced. At 6 equiv of Ag/MT, all of the original Cd^{2+} in the β-domain of Cd_7MT has been displaced and the only species present is $(Cd_4)^{\alpha}(Ag_6)^{\beta}MT$. Upon further addition of Ag^+, Cd^{2+} is displaced from the α-domain of $(Cd_4)^{\alpha}(Ag_6)^{\beta}$-MT to form $Ag_{12}MT$. From the amounts of Ag^+ required to bring about these changes, it is inferred that homogeneous Ag_6-clusters are being formed in both domains and that binding of Ag^+ to the β-domain is significantly stronger than to the α-domain.

The only evidence for the production of any species besides those depicted in Scheme 1 is the small resonance that appears during the titration at 632 ppm (Figure 1). The origin and significance of this resonance is not known. Its intensity increases to about 10% of that of the original Cd signals during the displacement of the β-domain Cd^{2+} ions, but does not change thereafter. In fact, it remains even after all of the original resonances disappear (data not shown). One possibility is that the resonance arises from a particularly stable minor form of a Cd,Ag mixed-metal cluster in the β-domain in which Cd occupies a single site in a cluster containing Ag at all of the other sites. Another possibility is that the extra resonance comes from an oxidized form of Ag,Cd-containing protein, in which a single Cd has a different environment. DEAE HPLC experiments have also detected a minor product that may be the source of the extra Cd resonance (Li & Otvos, 1996).

Just as it was found previously that CdMT and ZnMT cannot co-exist in solution without undergoing an intermolecular metal exchange reaction leading to the production of mixed-metal species (Nettesheim et al., 1985), CdMT and AgMT were shown here to undergo the same type of reaction. In Figure 3, the reduction in the intensities of the peaks corresponding to the β-domain 3-metal cluster coincides with an increase in the intensities of the peaks corresponding to the α-domain 4-metal cluster when Ag_{12}-MT is added to Cd_7MT, indicating that a metal exchange reaction between the two proteins has occurred. The reaction pathway representing this process, in which Ag^+ migrates from α- to β-domains in exchange for Cd^{2+}, is depicted in Scheme 2.

Scheme 2: Proposed Pathway of the Interprotein Metal Exchange Reaction between Cd_7MT and $Ag_{12}MT$



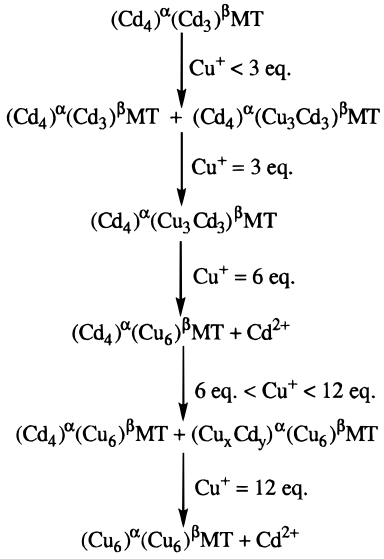
Assuming that the metal binding stoichiometries in the two domains are correctly represented and that partially-occupied clusters are disfavored compared to those containing their full complement of metal, the movement of six Ag^+ ions out of the α-domain of a $Ag_{12}MT$ molecule in exchange for three Cd^{2+} ions will create a situation in which some metal-deficient MT is produced. As shown in Scheme 2, 25% of the original $Ag_{12}MT$ will end up with no α-domain metal ions, represented as $(apo)^{\alpha}(Ag_6)^{\beta}MT$. Presumably, the unliganded cysteine sulfurs in this species would become susceptible to oxidation reactions, which might be responsible for the species giving rise to the minor peak at 632 ppm observed in Figures 1 and 3.

Another prediction from Scheme 2 is that complete elimination of ^{111}Cd NMR signals from the β-domain of Cd_7-MT should require the addition of one mole equivalent of $Ag_{12}MT$, which is what was observed in Figure 3. 75% increases in the intensities of the 4-metal cluster resonances would be expected if all of the $^{111}Cd^{2+}$ that was originally located in the β-domain of Cd_7MT was transferred to the α-domain of $Ag_{12}MT$. In fact, as shown in Figure 4, the 4-metal cluster resonances were seen to increase in intensity by about 40%. The failure to achieve the full 75% intensity increase predicted by Scheme 2 is probably due to the fact that the Cd_7MT starting material used in this experiment did not have its full complement of β-domain Cd, as indicated by the lower than normal amplitudes of the 3-metal cluster resonances seen in the bottom spectrum of Figure 3.

Taken together, the results of the reactions of Cd_7MT with both Ag^+ and $Ag_{12}MT$ indicate the existence of only one stable mixed-metal Cd,AgMT species, namely $(Cd_4)^{\alpha}(Ag_6)^{\beta}$-MT. The driving force for the production of this species must be the thermodynamic stability gained by having Ag^+ and Cd^{2+} occupy exclusively β- and α-domain clusters, respectively, rather than be relegated to bind simultaneously to both domains.

From the results in Figures 5 and 6, it is clear that Cu^+ shares with Ag^+ a strong preference for binding to the β-domain of MT and, like Ag^+, 12 equiv of Cu^+ are needed

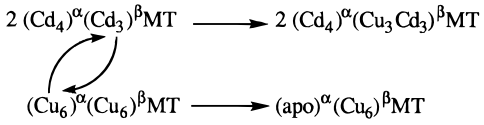
Scheme 3: Proposed Pathway for the Metal Displacement Reaction of Cd_7MT with Cu^+



to displace all of the Cd^{2+} from Cd_7MT. There were, however, some interesting differences observed in the behavior of Cu^+ compared to Ag^+. In the Ag^+ titration of Cd_7MT (Figures 1 and 2), the intensities of the 3-metal cluster Cd resonances were seen to progressively decrease until they disappeared at a Ag/MT ratio of 6. During this process, there was a linear relationship between the amount of Ag^+ added and the observed decrease in 3-metal cluster resonance intensity. Parallel measurements of the Ag and Cd concentrations in the NMR samples determined by AAS confirmed that for each Ag^+ ion added, approximately 0.5 equiv of Cd^{2+} was displaced and removed from solution by the Chelex-100. In the corresponding Cu^+ titration (Figure 5), it was found that only 3 Cu^+ ions were needed to eliminate the original 3-metal cluster Cd resonances (peaks II, III, and IV). Concurrently, several new Cd resonances appeared (peaks 1, 2, and 3) and their intensities maximized at a Cu/MT ratio of about 3. In further contrast to the behavior of Ag^+, metal analysis indicated that the first 3 equiv of Cu^+ became bound to the protein without displacing any Cd^{2+}. Only when Cu^+ in excess of 3 equiv was added was Cd^{2+} actually removed from the protein. Similar behavior was also observed later in the titration as Cu^+ became bound to the α-domain. Following addition of 10 equiv of Cu^+, all of the *original* 4-metal cluster Cd signals had been eliminated, but at least five new resonances had taken their place. These in turn disappeared upon the addition of about 12 equiv of Cu^+.

We interpret the results of the Cu^+ titration data according to the reactions shown in Scheme 3. Up to about 3 equiv of added Cu^+, Cd^{2+} is not displaced from the *β*-domain but rather becomes a constituent of one or more uncharacterized mixed-metal clusters, depicted for simplicity as a $(Cu_3Cd_3)^{\beta}$ cluster. Since the environments of the Cd^{2+} ions in the *β*-domain are no longer the same as in the original $(Cd_3)^{\beta}$ cluster, the 3-metal cluster resonances (peaks II, III, and IV) disappear and a number of new resonances (peaks 1, 2, and 3) appear from the Cd^{2+} ions in the mixed-metal cluster species. Other Cu,Cd mixed-metal clusters have also been proposed by other studies (Li & Weser, 1992). As additional Cu^+ is added, these new signals disappear as the mixed-metal *β*-domain clusters are replaced by $(Cu_6)^{\beta}$ clusters and Cd^{2+} ions are displaced from the protein. At a stoichiometry

Scheme 4: Proposed Pathway of Interprotein Metal Exchange between Cd_7MT and $Cu_{12}MT$ at CuMT/CdMT ≤ 0.5



of about 6 equiv of Cu^+, the ^{111}Cd NMR spectrum again becomes very simple with only resonances appearing from the Cd^{2+} in the intact α-domain Cd_4-cluster of $(Cd_4)^{\alpha}(Cu_6)^{\beta}$-MT. Addition of the next 3−4 equiv of Cu^+ produces a similar sequence of events, with the Cd^{2+} in the α-domain presumably being converted to one or more mixed-metal species [depicted as a $(Cu_xCd_y)^{\alpha}$ cluster) prior to becoming displaced concurrent with formation of the $(Cu_6)^{\alpha}$ cluster.

Because of frequent reports of variability in Cu-binding stoichiometry, possibly caused by cysteine oxidation and/or disproportionation of Cu^+ in aqueous solution (Li & Weser, 1992), we repeated the Cu^+ titration experiments many times and took special precautions to avoid oxidation. For example, the Cu^+ stock solution was confirmed by ESR and lack of reaction with BCS (bathocuproine disulfonic acid) (Zak, 1958) to be free of detectable Cu^{2+} (data not shown). All sample manipulations were carried out in an anaerobic chamber under nitrogen atmosphere, and NMR experiments were performed using sealed NMR tubes. In addition to using $[Cu(CH_3CN)_4]ClO_4$ as the source of Cu^+, titrations were also carried out using freshly-made CuCl in the presence of reducing agents such as glutathione and ascorbate. In all cases we consistently found that the Cd NMR signals from the $(Cd_3)^{\beta}$ cluster were abolished upon the addition of 3−4 equiv of Cu^+ and those from the $(Cd_4)^{\alpha}$ cluster upon the addition of 8−10 equiv of Cu^+ (data not shown). Thus, we do not believe that the differences found in the behavior of Ag^+ and Cu^+ are attributable to the greater oxidative susceptibility of Cu^+.

In the direct intermolecular metal exchange reaction between Cd_7MT and $Cu_{12}MT$, spectral changes (Figure 6) very similar to those seen in the early stages of the Cu^+ titration experiment (Figure 5) were observed. Increasing amounts of $Cu_{12}MT$ added to Cd_7MT brought about a progressive decrease in the size of the 3-metal cluster resonances and the appearance and simultaneous increase in the intensity of the three new resonances attributed to a *β*-domain mixed-metal cluster species. In contrast to the results of the reaction of Cd_7MT with $Ag_{12}MT$, no significant increases in the intensities of the 4-metal cluster signals were observed to accompany loss of the 3-metal cluster signals. In addition, the amount of $Cu_{12}MT$ required to bring about elimination of the 3-metal cluster peaks was about 0.5 equiv of compared to the roughly 1 equiv of $Ag_{12}MT$ required to accomplish the same thing. In Scheme 4 is presented the reaction pathway postulated to bring about the NMR spectral changes observed in Figure 6. The 6 Cu^+ ions from the α-domain of one $Cu_{12}MT$ molecule furnish the Cu^+ needed to produce the mixed-metal *β*-domain species in two other Cd_7MT molecules. As a result of this reaction, there would be expected to be no change in the intensities of the 4-metal cluster resonances, as was observed experimentally. If the experiment in Figure 6 had involved addition of more than 0.5 equiv of $Cu_{12}MT$, an increase in the size of the 4-metal cluster resonances would have been anticipated, accompanied by a loss in intensity of the three new mixed-metal *β*-domain

signals as $(Cd_4)^{\alpha}(Cu_3Cd_3)^{\beta}$MT was converted to $(Cd_4)^{\alpha}(Cu_6)^{\beta}$-MT.

Despite apparent differences in their abilities to form stable mixed-metal cluster species, Ag^+ and Cu^+ display many similarities in their interaction with MT that support the use of Ag^+ as an NMR-active analog of Cu^+ in future structural studies. Both Ag^+ and Cu^+ bind to MT much more strongly than Cd^{2+}, both bind more strongly to the β-domain than to the α-domain, both bind with a stoichiometry of 12 equiv to form all-Cu or all-Ag species, and both form stable hybrid MT species containing $(Cd_4)^{\alpha}$-domains and $(Cu_6)^{\beta}$- or $(Ag_6)^{\beta}$-domains.

The two domains in MT have been proposed on the basis of the crystal structure to be functionally independent units (Robbins et al., 1991). However, since the connecting peptide between the domains is quite short (residues 30−32), it is not known how much influence the structure of the cluster in one domain might have on the other. The results presented here indicate that the ^{111}Cd chemical shifts of the Cd^{2+} ions in the α-domain are completely unaffected by the binding of either Ag^+ or Cu^+ to the β-domain. We conclude that the two domains are indeed structurally independent in solution and that the type of metal bound to one domain does not appreciably influence the structure or properties of the other domain. This would be in accord with the idea that the two domains of MT may have evolved to enable the protein to function simultaneously in the metabolism of both monovalent and divalent metal ions. Since Cu^+ binds much more tightly than Zn^{2+} or Cd^{2+} to *both* domains of MT and can rapidly displace divalent metals from their native binding sites, Cu^+ binding has the potential to disrupt the essential role(s) played by MT in zinc metabolism. The strong thermodynamic preference of the β-domain for binding monovalent metal ions, plus kinetically-accessible pathways for movement of monovalent metals from the α-domain of one MT molecule to the β-domain of another *via* intermolecular metal exchange, might provide an effective mechanism of keeping Zn and Cu partitioned in separate structural domains to allow each to function independently in essential metabolic processes unimpeded by the presence of the other.

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