Proteolytic studies of rat liver metallothionein reconstituted in vitro with Cu salts revealed that the 2 metal centers fill in an ordered fashion. The B cluster in the NH₂-terminal β domain fills prior to Cu binding in cluster A. This is in contradistinction to cluster formation induced by the binding of Cd or Zn ions in which cluster A is the center of initial binding. The formation of metal cluster B by Cu occurs in a cooperative fashion yielding a saturated cluster with approximately 6 Cu⁺ ions bound. The B cluster is saturated with Cd or Zn after binding of only 3 metal ions. The preferential binding of Cd and Cu to the α and β domains, respectively, and the tolerance toward protolysis of these 2 different half saturated molecules permit the isolation of each domain. The metal cluster in each isolated domain can be reversibly formed with predicted stoichiometries of Cd and Cu. The folding of the polypeptide therefore appears to create each cluster independently. The metal binding data suggest that Cu-metallothionein contains 11–12 Cu ions, 6 bound in the β domain and 5–6 in the α domain. In contrast, Cd-metallothionein contains 7 Cd ions, 3 bound to β and 4 to α.

MT is a 61-residue polypeptide found in most eukaryotic cells that is capable of binding a variety of metal ions (1–3). The physiological function of the metalloprotein is unknown, but it has been suggested that it exists as a detoxification system against certain heavy metal ions or alternatively is involved in Zn and Cu homeostasis (4–6). The number of binding sites for Zn or Cd is 7 and these sites are ligated exclusively by 2 cysteinyl residues (1, 7, 8). The positions of these 20 cysteines in the amino acid sequence are invariant in mammalian MT isoforms (1, 2). Two polymorphic variants of MT (designated MT I and MT II) are induced in rodents by administration of metal salts or glucocorticoids (11–14). The two isoforms from the livers of rat and mouse differ in 12 and 15 positions of the 61-residue polypeptide, respectively (16, 17). Metal chelation appears to alter the structure from a random coil to a more compact conformation (9). Single crystals of Cd,ZnMT from rat liver have been grown for structural determination by x-ray diffraction analysis (10).

The isoforms induced by Cd invariably contain bound Zn with the average composition being 5 Cd and 2 Zn ions/molecule. The 7 metal ions are distributed in 2 separate polynuclear metal-cysteine clusters (18, 19). One center (cluster A) contains 4 metal ions bound to 11 cysteines, 5 of which probably exist as thiolate bridges. The other center (cluster B) binds 3 Cd or Zn ions through 9 cysteines and probably 3 thiolate bridges (18, 19). The 2 clusters are enfolded by separate domains of the MT molecule (20, 21). The 3-metal center (β domain) comprises the NH₂-terminal half of the molecule and the 4-metal cluster (α domain) is formed by the COOH-terminal half. The 2 clusters differ in their metal-binding properties. Results from ¹¹⁷Cd NMR experiments (18) and our isolation of the α domain (20) reveal that the metal ions in Cd,ZnMT are arranged such that cluster A contains 4 Cd ions and cluster B contains on the average 2 Zn ions and 1 Cd ion. We recently demonstrated by metal ion reconstitution studies that Cd and Zn binding was ordered (22). Binding initially occurs in cluster A followed by binding in cluster B. The binding process in each domain is cooperative, but no cooperativity is apparent between domains. Cd₄ε was shown to be a stable intermediate both kinetically and thermodynamically.

When rabbit liver MT is renatured with Co²⁺, metal cluster formation occurs in a random manner (23, 24). The first 4 metal ions bound produced no magnetic interaction (23, 24). Since Co-saturated clusters, i.e. Co-MT, are diamagnetic due primarily to antiferromagnetic coupling, the first 4 metal ions were presumably distributed between the 2 clusters. The remaining 3 Co ions gave increasing interaction as the separate sites became linked into the polynuclear centers.

Copper ions can also induce formation of MT and can also bind to the molecule (25, 28). Purification of MT induced by Cu in rat liver showed that 9 to 11 g atoms of Cu were complexed per mol of protein with the Cu ions being diamagnetic Cu⁺ (25). Molar ratios of cysteine to metal of approximately 2:1 have been observed in CuMT from various species (25–28) whereas a ratio of 3:1 is observed with Cd or ZnMT. The higher binding stoichiometry in CuMT compared to Cd,ZnMT was substantiated by metal displacement studies in which 1.5 eq of Cu⁺ were necessary for the displacement of Zn²⁺ in ZnMT (29). Since CuMT appears to have at least 10 sites for Cu⁺, we were interested in determining how the sites were distributed between the 2 domains and whether Cu binding was an ordered process.

**MATERIALS AND METHODS**

Supplies and reagents were obtained as previously described (20). The isolation of MT and the preparation of apo-MT were carried out as previously stated (20, 22).

Reconstitution studies were performed by addition of metal ions to metal-free thionein in 0.01 N HCl enclosed in an argon-purged glove bag at 25 °C. Solutions were degassed before use. The metal-MT mixture was neutralized to pH 7.5 by the addition of potassium phosphate or Hepes buffers. The final buffer concentration varied.

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¹ The abbreviations and designated terms are: MT, metallothionein; α, the COOH-terminal domain of metallothionein which encompasses cluster A; β, the NH₂-terminal domain containing cluster B; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

between 100 and 150 mM depending on the amounts of apoprotein used. In certain reconstitution experiments, subtilisin (protease:MT ratio of 1:30 by weight unless stated otherwise) was added to the anaerobic samples and digestion was allowed to proceed at 37 °C for 2 h in argon-purged sealed tubes. Proteolytic degradation of apo-MT at 37 °C is complete in a 30-min incubation period.

In experiments utilizing Cu\(^{2+}\), CuC\(_2\) was dissolved in 0.1 N HCl containing 4% NaCl by weight. The acid solution was exhaustively deaerated prior to the addition of CuC\(_2\). After preparation of the solution, an aliquot was withdrawn for determination of the Cu concentration. Metal analysis was performed on a Perkin-Elmer 505 A spectrometer. Protein concentrations were established by quantitative amino acid analysis and thiol titrations using dithiodipyridine in 0.2 M sodium acetate, pH 4, containing 1% sodium dodecyl sulfate and 1 mM EDTA. The millimolar extinction coefficient for 2-thiopyridine is 7.06 at 343 nm (30).

The individual domain polypeptides comprising individual domains from a single isoform were prepared by mild proteolysis of partially reconstituted protein molecules. The \(\alpha\) domain polypeptide was isolated after reconstitution of apo-MT with 4 eq of CdCl\(_2\) followed by digestion with subtilisin. After a 2-h incubation, the sample was chromatographed on Sephadex G-75 (superfine). The K\(_g\) 0.7 Cd peak was pooled and lyophilized. Apo-\(\alpha\)-MT was prepared by acidifying the sample to pH 2 followed by desalting by dialysis on Sephadex G-25, equilibrated in 0.01 N HCl. The \(\beta\) domain was isolated from proteolysis of Cu\(^{2+}\)-reconstituted MT. The K\(_g\) 0.7 peak from Sephadex G-75 was recovered and reduced with 0.1 M \(\beta\)-mercaptoethanol. The sample was then acidified to pH 0.5 followed by desalting on Sephadex G-25 equilibrated in 0.01 N HCl. The metal-free polypeptides were stored in 0.01 N HCl at -80 °C. Protein concentrations were determined by amino acid analysis.

The proteolytic protection assay was performed by quantitating the amino groups in the proteolysed reconstituted samples. Equivalent aliquots of each sample were reacted with fluorescamine in 0.2 M sodium borate, pH 8.6, and the fluorescence emission at 475 nm was measured on a Perkin-Elmer 650-10 S spectrophotometer after excitation at 390 nm (31). The quantity of protein typically used in an assay was 5 \(\mu\)g.

Polyacrylamide gel electrophoresis was performed at pH 8.9 in 7.5% gels (32). Analytical gel filtration was carried out on Sephadex G-75 (superfine) in 10 mM potassium phosphate, pH 7.8. Blue dextran and NaCl were included in samples to mark the excluded and internal volumes, respectively. Elution positions were determined and calculated as distribution coefficients (K\(_d\)).

**RESULTS**

Under our conditions of reconstitution, Cd and Zn ions reconstituted apo-MT to yield a metalloprotein that exhibits properties similar to those of native MT (22). As the occupancy of the binding sites approaches saturation, a greater protection against proteolysis is observed. This experimental approach was used in the present investigation to determine the order of cluster formation and stoichiometry of Cu binding to MT. Rat liver apo-MT I samples reconstituted with increasing equivalents of CuC\(_2\) were subjected to proteolysis for 2 h. The proteolyzed samples were then electrophoresed on non-denaturing polyacrylamide gels to resolve the fragments. Renaturation with low levels of CuC\(_2\) (1 to 5 Cu ions/MT molecule) resulted in 2 prominent bands with mobilities of 0.35 and 0.6. When 2 bands with identical mobilities were observed with each MT isoform, whereas native MT isoforms I and II show electrophoretic mobilities of 0.42 and 0.6, respectively. Integration of densitometric scans of the gels showed that the quantity of both fragments increased as the Cu equivalency approached 5 (Fig. 1). The R\(_F\) 0.63 band reached a maximal concentration near 5 Cu eq, whereas the band of R\(_F\) 0.35 from MT I peaked in intensity between 5 and 6. Quantification of the R\(_F\) 0.35 band in proteolyzed isoform II in the samples containing Cu in excess of 6 eq was not possible due to interference from a stain smear seen near the gel top. Beyond 6 Cu ions/molecule, the 2 stained bands in isoform I decreased in magnitude until they were no longer visible in the sample containing 7 Cu ions. The only stained component seen in isoform I samples with 7 or more Cu eq as either Cu\(^{2+}\) or Cu\(^{+}\) was a band that did not penetrate into the resolving gel, suggesting an aggregated or polymerized polypeptide. Similar aggregated bands were also visible in isoform II samples containing 7 or more Cu eq.

In attempts to identify what segment of metallothionein was rendered resistant to proteolysis by Cu binding, apo-MT reconstituted with 1 to 5 Cu ions/molecule were incubated with subtilisin and subsequently chromatographed on Sephadex G-75 equilibrated and eluted with 10 mM potassium phosphate, pH 7.8. The effluent was monitored by measuring both the Cu concentration and absorbance at 230 nm. Only 1 prominent peak with a K\(_d\) of 0.69 was observed in the elution fractions of samples renatured with 1, 2, and 4 eq (Fig. 2). The recovery of ultraviolet-absorbing material increased with higher Cu/MT molar ratios. Correspondence was observed between Cu concentration and absorbance values. Under identical conditions, native rat liver MT isoforms I and II elute with K\(_d\) values of 0.55 and 0.535, respectively (33), and Cd and II elute near K\(_d\) 0.7.

The Cu-containing polypeptide fraction from each MT isoform was pooled and subjected to acid hydrolysis. The amino acid compositions of these fractions are shown in Table 1 along with the compositions for the NH\(_2\)-terminal domains of MT as compiled from sequence analysis. The data demonstrate that 5 eq of Cu protect the \(\beta\) domain of each MT isoform from proteolysis. It is apparent from sequence data that the net electronic charge of the \(\beta\) domain polypeptide from the 2 isoforms is similar near neutral pH. In contrast, the net charge for the \(\alpha\) domain polypetide differs for the 2 isoforms as it has an additional negative charge. The similar electrophoretic mobilities for the proteolyzed Cu\(^{2+}\)-reconstituted MT samples from the 2 isoforms substantiates the identification of these bands as \(\beta\) domain fragments.

In order to determine whether the 2 stained bands seen on electrophoretic gels were both \(\beta\) polypeptide variants, the proteolyzed Cu\(^{2+}\)-renatured MT purified by gel filtration or anion exchange chromatography yielded the same two bands and the ratio of the quantitated bands was similar to that seen in Fig. 1 for the Cu\(^{2+}\)-renatured MT sample. Cu-reconstituted samples that had been digested with subtilisin were also chromatographed on DEAE-cellulose to determine whether \(\beta\) charge variants were apparent by an alternative procedure. Only a single prominent peak was observed in the effluent of the salt gradient. The single polypeptide which eluted on anion exchange was resolved into 2 bands by electrophoresis with similar R\(_F\) to the bands in Fig. 1, suggesting that the additional band possibly is generated by the electrophoretic conditions and, therefore, the characterization of the bands was not critical for our studies.

Reconstitutions were performed at varying dilutions to determine whether Cu\(^{2+}\) is a favored kinetic as well as thermodynamic intermediate. If binding occurred in a random fashion along the polypeptide, proteolysis may result in metal ion rearrangement that preferentially protects the \(\beta\) domain. Apometallothioneins I and II were renatured with 5 Cu eq and incubated in concentrations ranging from 1.6 to 160 \(\mu\)M with a fixed concentration of subtilisin (4 \(\mu\)M). Analysis of the digestion products on polyacrylamide gel electrophoresis revealed the same banding pattern with each isoform regardless of the dilution. In control incubations, autolysis products from subtilisin did not interfere in the \(\beta\) banding. Products were also evaluated by gel filtration. The same \(\beta\) polypeptides as demonstrated by amino acid analysis were observed to elute with a K\(_d\) near 0.69. The probability of metal ion rearrangement would be diminished by the dilution.
Copper Clusters in Metallothionein

FIG. 1. Integration of Coomassie-stained protein bands after non-denaturing polyacrylamide gel electrophoresis of apo-MT I and II reconstituted with 0 to 6 Cu eq. The gels (30 μg of sample/gel) were scanned, and the areas under the RF 0.35 (○) and RF 0.63 (●) peaks were quantified by weighing the cut-out peaks. The inset shows the actual gels for isoform I.

Cuβ was still observed in a similar yield, cluster B must be the site of initial Cu ligation.

The smearing seen on electrophoresis of samples with a high Cu equivalency suggested that aggregation may be occurring. Cu⁺ is known to catalyze the oxidation of thiols, so the polymerization of CuMT is probably due to intermolecular disulfide bond formation (34). In vitro renaturation of MT with Cu²⁺ salts has been shown to result in the reduction of cupric ions with the concomitant oxidation of cysteine thiols (35, 36). CuMT formed in vivo is known to bind Cu in a diamagnetic Cu⁺ state via a reduced thiol ligand field (25, 37). Apo-MT reconstituted with Cu⁺ shows initial binding within the β domain forming a colorless product. Titration of the thiols revealed an inverse relationship between the number of titratable thiols and the Cu equivalents added. Twenty thiols could be titrated in apo-MT with dithiodipyridine whereas the number decreased to 10 as the Cu²⁺/protein ratio reached 10. In order to avoid oxidation, reconstitution experiments were also carried out with Cu⁺ stabilized as CuCl₂ in acid. CuMT formed with Cu⁺ revealed a full complement of titratable sulphydryl residues. Electrophoresis and chromatographic studies showed that Cu⁺, like Cu²⁺, preferentially protects the β domain against proteolytic digestion. As the Cu⁺/protein ratio exceeded 5, aggregated species could also be seen upon electrophoresis or chromatography suggesting that the Cu-metallothionein ligand field is susceptible to oxidation.

The stoichiometry of Cu binding was studied in two ways. In the first approach, proteolyzed samples reconstituted with varying amounts of Cu²⁺ or Cu⁺ were chromatographed on Sephadex G-75 and G-25. Sephadex G-75 elution fractions of β were assayed by Cu and amino acid analyses to determine the metal/protein ratios and yields. Regardless of the Cu equivalents added to apo-MT, as either Cu⁺ or Cu²⁺, the resulting metal content varied between 5 to 6 metal ions bound (Table II). The mean metal/protein molar ratios were 5.8 and 5.3 for Cu⁺ and Cu²⁺, respectively. The recovery based on starting material rose as the added Cu equivalency increased up to 6. The recoveries of β are comparable to those of α in Cd reconstitutions in which the binding of Cd is cooperative in nature (22). In resolution of Cuβ from proteolyzed fragments by Sephadex G-25 chromatography, samples reconstituted with 1, 3, and 5 Cu eq, yielded β recoveries of 15, 45, and 92%, respectively. Again, these yields are in agreement.
Amino acid composition of the \( \beta \) domains of metallothionein

The composition of \( \beta I \) is an average of 6 different preparations of the polypeptide, whereas \( \beta II \) is from duplicate samples.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Cu-renatured MT ( K_d = 0.7 ) peak</th>
<th>Rat liver MT residues 1-31 (Ref. 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Isoform I</td>
<td>Isoform II</td>
</tr>
<tr>
<td>Asp</td>
<td>2.9</td>
<td>2.9</td>
</tr>
<tr>
<td>Thr</td>
<td>2.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Ser</td>
<td>6.1</td>
<td>4.4</td>
</tr>
<tr>
<td>Glu</td>
<td>0.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Pro</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Gly</td>
<td>3.1</td>
<td>2.2</td>
</tr>
<tr>
<td>Ala</td>
<td>0.3</td>
<td>2.1</td>
</tr>
<tr>
<td>Cys*</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Met</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>Ile</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>Lys</td>
<td>4.4</td>
<td>4.9</td>
</tr>
</tbody>
</table>

\* Cys values determined by titration with 2,2'-dithiodipyridine.

FIG. 2. Gel filtration of subtilisin-digested Cu-renatured MT I. Apo-MT I samples (1 mg) were reconstituted with 1, 2, and 4 Cu eq as specified in the upper right corner of each profile. After a 2-h digestion with the protease, the samples were chromatographed on a Sephadex G-75 (superfine) column (2.5 x 110 cm) in 10 mM potassium phosphate, pH 7.8. The effluents were monitored for absorbance at 230 nm (○) and Cu concentration (●).

FIG. 3. Effect of Cd\(^{2+}\) and Zn\(^{2+}\) on the proteolytic susceptibility of apo-MT I. The protein (100 \( \mu g \)) was reconstituted with varying Cd equivalents as specified on the abscissa. After a 2-h incubation at 37 °C in the presence of subtilisin (1 \( \mu g \)), aliquots containing 5 \( \mu g \) of CdMT (●) and ZnMT (〇) were reacted with fluorescamine. The relative fluorescence (——) of the samples were measured. Aliquots containing 20 \( \mu g \) were diluted to 1 ml with 10 mM potassium phosphate for ultraviolet absorption spectroscopy. Absorbances at 250 nm for CdMT (●) and 220 nm for ZnMT (〇) samples were plotted (——).

Monomeric MT on Sephadex G-75. The yield was slightly higher if chromatography was carried out in the presence of 1 mM \( \beta \)-mercaptoethanol and \( N_2 \)-purged buffer. Analysis of the monomeric species revealed a Cu content that varied from 10.5 to 12.2.

The second approach used to establish binding stoichiometry was a proteolytic protection assay. Since apo-MT is digested to small peptides by subtilisin, whereas the holoprotein is completely resistant, the protein reconstituted with varying amounts of metal ion could be tested for its susceptibility toward proteolysis. The extent of digestion was monitored by the fluorescamine reaction which quantifies primary amino groups. Increasing the metal equivalency of either Cd or Zn from 0 to 7 resulted in a linear attenuation in the fluorescamine reactivity (Fig. 3). Concentrations of metal ion in excess of 7 did not affect any further changes. The absorption spectrum of MT is marked by prominent electron transfer transitions generated by metal ion chelation (7). Maximal absorbance of the reanimated samples was seen with 7 Cd or Zn ions (Fig. 3), a number in agreement with the binding sites...
on the protein for these two ions.

The protein isoforms renatured with Cu salts show a similar metal-dependent protection against proteolysis (Fig. 4). The number of Cu$^+$ equivalents necessary to render the molecule resistant was approximately 11, although the transition point was not as obvious as with Cd reconstitutions. The transition point in Cu$^{2+}$ reconstitutions exceeded 7, but the curved nature of the line made the exact identification difficult. The Cu$^{2+}$/thiol redox reaction necessarily complicates the assay.

The proteolysis assay was tested for effects of metal ions on subtilisin. Casein and ribonuclease were two substrates used to determine whether metal ions perturbed the action of subtilisin. Cd and Zn salts at concentrations exceeding those used in the present studies were without effect on the proteolytic digestion of casein or ribonuclease. CuCl$_2$ at a concentration equivalent to that in CuL$_2$ reconstitution samples resulted in a 35% inhibition of the proteolysis of casein. Higher Cu concentrations were necessary to observe the same inhibition of the subtilisin-dependent digestion of casein or ribonuclease when Cu was associated with metallothionein. Therefore, the effect of Cu on subtilisin would not invalidate the experimental approach.

The individual domain polypeptides were isolated for reconstitution studies. Using the proteolytic protection assay, $\alpha$ and $\beta$ were repeatedly rendered insensitive to proteolysis in the presence of 4 and 3 Cd or Zn ions, respectively (Fig. 5). Complete protection was achieved with Cu$^+$ at a stoichiometry near 5 ions/$\alpha$ or $\beta$ molecule. A similar content in $\beta$ was evident with Cu$^{2+}$ providing that 2 mM dithiothreitol was present in the incubation mixture. In the absence of the reductant, the apparent transition point was closer to 3 Cu/molecule. Binding was verified by observing co-elution of Cd and each polypeptide upon gel filtration. The actual Cu$^\beta$ ratio is probably 6. This is inferred from the observations that Cd$^\beta$ shows bands of the same electrophoretic mobility as Cu$^\beta$. The net charge computed for Cd$^\beta$ would equal Cu$^\beta$ if the latter binding stoichiometry was 6 Cu$^+$/Cu$^\beta$ molecule.

**DISCUSSION**

Reconstitution studies of apo-MT with Cu salts revealed that Cu ions bind preferentially to the $\beta$ domain of the molecule. The binding appears to occur in a cooperative fashion. Regardless of the equivalents of added Cu, the $\beta$ polypeptide is isolated with about 5.8 Cu ions bound/molecule. Cu$^\beta$ is a kinetic and thermodynamic intermediate in the binding process. Since binding of Cu renders the $\beta$ fragment insensitive to proteolysis, Cu$^\beta$ can be isolated by mild proteolysis. The order of cluster formation by Cu is opposite to that observed with Cd or Zn ions. In the latter case, binding initiates in the $\alpha$ domain in a cooperative process (22).

Cu is known to be bound in a diamagnetic cuprous state to reduced thios of MT (25, 37). Reconstitution with Cu$^{2+}$ leads to binding of cuprous ions, but the metal reduction is accompanied by sulfhydryl oxidation. Renaturation of apo-MT with Cu$^+$ stabilized anaerobically as CuCl$_2$, results in Cu$^+$ binding to titratable cysteines. However, the Cu$^+$/S-Cys complex formation in MT is susceptible to oxidation, since aerobic exposure of the metalloprotein to chromatography or electrophoresis leads to aggregation. Fetal Cu-MT has been shown to oxidize and yield polymeric species of the molecule (35).

Aged preparations of Cu-MT have also been reported to oligomerize, and this aggregation can lower the Cu content of the molecule (28, 39). This tendency of Cu-MT and CuS to oxidize necessitates reduction of the molecule prior to preparation of apo-$\beta$ for reconstitution studies.

The binding stoichiometry of Cu-MT differs from Cd, or ZnMT. Whereas Cd or ZnMT display saturation binding at 7 metal ion eq, Cu-MT appears to have approximately 10–12 sites. The Cu content was determined by 2 methods in reconstitution experiments. The number of Cu-binding sites determined is consistent with the Cu content observed in rat liver CuMT formed in vivo after Cu$^{2+}$ loading (25). The Cu ions bound to the rat liver MT were shown to be cuprous, suggesting specific binding. Whereas CdMT exhibits a cysteine/metal molar ratio of 3:1, CuMT from various species has been shown to have a ratio closer to 2:1 (25–28). Metal displacement studies of ZnMT suggested that 1.5 eq of Cu were necessary for complete Zn displacement (29). These findings are consistent with the Cu stoichiometry which we observed. The Cu content of fetal MT from human liver has been reported to be less than 3. The Cu-protein, unlike the fetal ZnMT, tends to be insoluble which suggests a polymerized state (40, 41).

The reduced Cu content of fetal CuMT is probably a result of the decreased binding affinity of the oxidized protein.

The $\alpha$ and $\beta$ domains of MT are known to complex 4 and 3 Cd ions, respectively (19–21). Purified domains, separated from each other, ligate the expected number of metal ions,
thereby becoming resistant to proteolysis. Thus, the 2 domains are independent in their folding and binding process. The beta domain appears to exhibit binding saturation near 6 Cu ions. Considering that the electrophoretic mobility of Cu Beta is the same as that of Cd, the Cu content would have to be 6 in order for the net charge of Cu Beta to be similar to Cd.

The number of Cu sites in the alpha domain also appears to be between 5 and 6. Summation of the binding stoichiometries of the individual domains (CuBeta + Cu26-Alpha = Cu12-13MT) is in good agreement with that observed for the intact molecule.

Metal ion stoichiometries and distribution of the ions along the polypeptide chain have been reported for 2 other Cu proteins. A 25-residue polypeptide that is homologous to the beta domain of MT exists in Neurospora crassa. The polypeptide contains 7 cysteynil residues and binds 6 Cu+ ions/molecule, apparently in a single cluster (42). The molar cysteine/metal ratio is closer to 1 than the ratio for Cu Beta in MT. Calf liver MT has been shown to contain the usual 2 clusters with 4 Cd-displaceable Zn ions in alpha and 3 Cu in beta (43). The reason for only 3 Cu in the B cluster is uncertain. We are currently investigating whether initial formation of a saturated A cluster imparts constraints in the folding of the beta domain which may limit the number of Cu sites.

The higher binding content of Cu MT compared to Zn MT may result in 2 different structural states of the protein. Preliminary evidence showing that the Stokes radii of the two metalloproteins differ also suggests that the conformations may be distinct. Since the protein appears to participate in the metabolism or homeostasis of both Cu and Zn in both adult and neonatal tissues, the cell may be able to discriminate between Cu and Zn forms of the protein by virtue of conformational differences. The observed variations between Cu MT and Zn,Cd MT are not the only differences in metal binding by MT. For example, the clusters in Co MT appear to fill in a noncooperative fashion (23). Such variations may be important not only in cellular recognition but conceivably may be relevant to the stability of the molecule and to the yet unresolved physiological function of metallothionein.

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