Distinct Metal-binding Configurations in Metallothionein*

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In a study of the binding stoichiometry of various metals to rat liver metallothionein, the protein appears to coordinate metals in 2 distinct configurations. Ions of at least 18 different metals were shown to associate with the protein suggesting that there is little specificity in binding. Most metals exhibited saturation binding at 7 mol eq forming M7-metallothionein. These included Bi(III), Cd(II), Co(II), Hg(II), In(III), Ni(II), Pb(II), Sb(III), and Zn(II). Others metals including Os(III), Pd(II), Pt(IV), Re(V), Rh(III), and Ti(III) give a positive indication of binding, but stoichiometries were unclear. Ag(I) and Cu(I) bound in clusters as M12-metallothionein. This binding stoichiometry was determined in 3 ways: (a) by determining the equivalence point in Cu- and Ag-titrated samples where resistance to proteolysis is maximal; (b) by determining the point where Zn ions are completely displaced from Zn7-metallothionein; and (c) by direct binding studies. Reconstituted protein, recovered from gel filtration, had an average Ag content of 11.5 g atoms/mol of protein. A similar stoichiometry for the Cu-protein resulted from displacement of Zn from Zn7-metallothionein by Cu(I). The M12-protein was converted to the M7-protein by displacement of Ag(I) or Cu(I) with 7 mol eq of Hg(II). Whereas the distribution of metals in the 2 domains of M7-metallothionein is M4α and M8β, the arrangement in the M12-molecule is probably M4α and M8β. We propose that metallothionein ligates Ag(I) and Cu(I) in a trigonal geometry by bridging thiolates. This is in contradistinction to a tetrahedral binding geometry in the M7-protein. Distinct binding configurations may result in different tertiary structures for M7- and M12-proteins which may relate to metabolic specificity of Zn-metallothionein and Cu-metallothionein, respectively.

Mammalian metallothionein is folded into 2 domains that form separate polynuclear metal-cysteine thiolate clusters (1-3). The 7 metal ions of Cd,Zn-metallothionein are distributed as a M5-Cys59 complex formed by the NH2-terminal β domain and a M2-Cys11 complex enfolded by the COOH-terminal α domain. The structure of the domains is dependent on the presence of metal ions. The apoprotein exists as a random coil (4). Metal binding induces a specific folded conformation consistent with a prolate ellipsoid that has an axial ratio of 6 (4-6). A molecular model of the protein indicates that the clusters are formed by a tightly wrapped polypeptide stabilized primarily by metal ligation and secondarily by β turns and H-bonding between side chain hydroxyl residues and amide carbonyls of the polypeptide backbone (7).

The metal ions in each cluster of Cd,Zn-metallothionein are tetrahedrally coordinated to 4 cysteine thiolates (5, 8). Eight of the 20 ligating cysteines probably exist as bridging sulfurs in the clusters (3, 5). Other metals with tetrahedral geometry in metallothionein complexes include Bi(III), Co(II), Hg(II), Ni(II), Pb(II), although the binding stoichiometry for some of these metal-protein complexes has not been established (8-12).

Although binding stoichiometries and coordination geometry have not been clearly established for Ag(I), Au(I), and Cu(I) (13-15), Cu-metallothionein is one form of the protein that deviates from the usual coordination of 7 tetrahedrally bound metals/polypeptide (15-17). We recently found that 11 or 12 Cu ions were bound to metallothionein and that the β domain can coordinate 6 Cu(I) ions, unlike the 3 Zn(II) ions/β domain in Zn-metallothionein (17). The higher apparent binding stoichiometry of Cu-metallothionein suggests that the Cu-protein adopts a conformation different from that of Cd,Zn-metallothionein. Cu(I) can form tetrahedral complexes but usually does not with sulfur ligands (18). Several small Cu-S complexes with Cu5S6 and Cu6S7 cores have been characterized, and the usual coordination chemistry is trigonal Cu(I) ions with bridging sulfurs (19-22).

It is important to understand the coordination properties and structure of both Zn-metallothionein and Cu-metallothionein, because the protein may function in cellular processes involving both Zn and Cu (23-28). These two forms of metallothionein may play a central role in intestinal absorption, intracellular storage, transport, or excretion of Cu and Zn. Although Zn-metallothionein appears to be the predominant form of metallothionein in adult human tissues, Cu-metallothionein may play a central role in the estimation of the binding stoichiometry of the Cu-protein would aid in the estimation of the binding stoichiometry. In addition, we wanted to determine the selectivity of metal binding to the protein and to find the range of binding stoichiometries. In the present report, evidence is provided that ions from at least 18 different metals associate with the protein. Although the prevalent binding configur-
tion is the M₇-protein, Ag(I) coordinates to the protein similarly to Cu(I) with a equivalency of about 12.

**MATERIALS AND METHODS**

**RESULTS**

**Ultraviolet Absorption Assay**—In order to study the specificity of metal binding to metallothionein, in vitro metal reconstitution of apometallothionein was used. The procedure involved anaerobic titration of the apoprotein with increasing mol eq of various metal ions. It is well established that renaturation of apometallothionine with Cd(II) and Zn(II) restores properties characteristic of native Cd,Zn-metallothionein (5,37). One characteristic feature of the metalloprotein is the prominent absorption spectrum from 240 to 300 nm which results from metal-ligand charge-transfer transitions. The shape and extinction coefficients of the spectrum are distinctive for different metals bound to the protein (8-13, 38). Increasing the metal equivalency in subsaturating amounts in metallothionein samples resulted in an increase in the absorbance (36,37).

Various metal ions were screened for their ability to enhance the ultraviolet absorption of apometallothionein. Only a few transition and group III to V cations (Cr(III), Mn(II), Ge(IV), Ti(II), and Zr(IV)) were without effect. Group II divalent ions (Mg, Ca, Sr, Ba) had no effect. Metal ions that yielded a significant increase in ultraviolet absorption as a function of added metal were tested in a second experiment. Renaturation studies were performed with both intact apometallothionein and proteolyzed apoprotein. Metals (Cd(II), Zn(II), and Cu(I)) ligated in chelated clusters showed greater absorption compared to ligation by cysteines from the proteolyzed protein. If binding of a particular metal occurs by only uninnuclear thiolates, little difference may be observed in comparing intact to proteolyzed metallothionein. In Table I the ultraviolet absorbance difference is shown for these 2 states of the protein mixed with various metals. Several cations (Fe(II), Fe(III), Ir(III), W(VI)) increased the absorbance of proteolyzed apometallothionein to about the same extent as the intact protein. Other metal ions augmented the ultraviolet absorbance of intact metallothionein more appreciably compared to their effect on the digested protein. One interpretation of the data is these latter metals may be associating with the protein in polyincular clusters analogous to Cd, Zn, and Cu ions.

**Proteolytic Protection Assay**—Apometallothionein is digested to small peptides by proteases (subtilisin, proteinase K) whereas the native metalloprotein is completely resistant. We demonstrated previously that metallothionein renatured with 7 mol eq of Cd(II) or about 12 mol eq of Cu(I) was resistant to incubation with these proteases (15,36). Various other metal ions were tested for their ability to protect the protein against proteolysis (Table II). The extent of digestion was monitored by reaction with fluorescamine, which quantifies primary amino groups. Metallothionein protected by a metal from proteolytic digestion has a fluorescamine fluorescence yield similar to that of the protein in the absence of a protease. The protein reconstituted in the presence of 10-12 mol eq of Ag(I), Bi(III), Cd(II), Cu(I), In(III), Ni(II), Os(III), Pb(II), Pd(II), Rh(III), Sb(III), TI(II), and Zn(II) was either completely or largely resistant to the proteases. Only partial protection was afforded by Fe(II), Fe(III), Ir(III), Pt(II), Pt(IV), and Sn(II), although higher metal ion concentrations of Fe(III), Mn(II), and Sn(II) enhanced the resistance. Al(III), Ba(II), Ca(II), Cr(II), Ga(III), Ge(IV), K(I), Mg(II), Mn(II), Mo(VI), Sr(II), V(IV), W(VI), and Zr(IV) were without significant effect at 30 mol eq. The results refer only to apparent protection, since resistance would also be apparent if the metal inhibited the activity of the proteases. The metals were tested for protease inhibition in a proteolysis reaction using casein and ribonuclease as substrates for subtilisin and proteinase K. Most of the cations were without significant effect on the proteases. Addition of Au(III), Cu(I), Hg(II), Os(III), Pd(II), Re(V), Rh(III), and Ru(III) in concentrations equivalent to those used in the studies with metallothionein resulted in partial inhibition of one or both of the proteases. If the metals are associated with metallothionein, greater concentrations of the metal were found necessary to affect the activity of the proteases. Decreasing the concentration of the protease 3-fold did not alter the results, so a partial inhibition of one enzyme should not affect the outcome.

The apparent metal-dependent protection against proteolysis was also checked by analysis of the peptides on thin layer chromatography. Numerous peptides were visualized by ninhydrin staining after digestion of apometallothionein with either subtilisin or proteinase K. Only a few faint bands were observed other than the native protein which was at the origin when the protein reconstituted with Ag(I), Cd(II), Cu(I), In(III), Ni(II), Pb(II), Pd(II), Sb(III), TI(III), and Zn(II) was incubated with one of those proteases.

There are numerous factors which influence the proteolysis assay resulting in different optimal conditions for certain metals. Besides the obvious metal-anion solubility effect with certain buffers, the pH of the reaction is an important variable because of displacement of complexed metal ions by protons. With certain metals the protection decreased as the pH was lowered. Ions of Zn(II), Pb(II), In(III), and Cd(II) became ineffective in protecting metallothionein from proteolysis by pepain at pH values of 4, 3, 2.5, and 2.5, respectively. Whereas the protection rendered by Bi(III), Cd(II), Cu(I), Hg(II), and Pb(II) was insensitive within the pH range of 5.5 to 7.8, the resistance of the molecule reconstituted with In(III), Sb(III), or Hg(II) increased as the pH was lowered from 7.8 to 5.5. This effect appears to result from the pH-dependent instability of the aqueous metal ion due to hydrolysis (39). Support for this interpretation came from other experiments. In the case of Sb(III), the extent of protection at pH 7.8 became almost complete as the metal ion concentration was increased to 60 mol eq. Cation aqueous stability was also apparent when the order of addition of the metal ion and buffer was varied. When the reconstitution procedure was altered so the apoprotein was neutralized to pH 7.8 prior to addition of the metal ion, Sb(III) and In(III) became ineffective in exerting a protective effect on the protein. Conversely, only a slight effect was observed in preneutralizing the apomolecule to pH 7.8 and using Cd(II) or Bi(III) as the protecting metals. At pH 5.5 a concentration of In and Sb of 10 mol eq was adequate for complete proteolytic resistance. The pH results with In(III) and Sb(III) were confirmed by peptide analysis using thin layer chromatography.

The protease insensitivity of metallothionein reconstituted with various metals is also affected by the time of incubation. Resistance to proteinase K at pH 7 of the molecule containing

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1 Portions of this paper (including "Materials and Methods," Tables I-IV, and Figs. 2, 5, 7, and 8) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Chemical Education, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M:2897, cite the authors, and include a check or money order for $5.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
bound Ag(I), Cu(I), Cd(II), Pb(II), Ru(III), or Rh(III) was unchanged for times up to 40 h. To the contrary, the protein coordinating Bi(III), Hg(II), In(III), Ni(II), Sb(III), Sn(II), or Zn(II) became increasingly sensitive to digestion after prolonged time under similar conditions. Although this liability may be related to the structure of the metalloprotein, aqueous instability of certain metal ions at pH 7 may also be a contributing factor.

The proteolytic protection assay was also used to assess binding stoichiometry with various metals. The procedure was good since the incubations could be performed anaerobically and large numbers of samples could be screened. Titrations of apo metallothionein with increasing metal equivalents led to a steady attenuation in the fluorescence resulting from fluorescamine reaction with amino groups until a value was reached that was similar to that of the native protein. The assumption is that maximum protection against proteolysis is achieved only upon saturation of all binding sites. In previous experiments with Cd(II) and Zn(II), the equivalency at the break point in the fluorescence curve was 7 which is known to be the maximal number of metals bound (15). Titrations of the protein with Bi(III), In(III), Ni(II), Pb(II), Pd(II), and Sb(III) revealed a minimum in the fluorescence curves at 7 mol eq (Figs. 1 and 2). A similar number was observed when the maximal ultraviolet absorbances of many metal-protein complexes (Cd(II), Hg(II), In(III), Pb(II), Sb(III), and Zn(II)) were monitored. With certain metals (Bi(III) and Pd(II)) the metal ions in the buffered solutions absorbed ultraviolet light, thereby masking a transition point. In the titrations with Hg(II), the absorbance curve at 250 nm was complicated by a drop in the absorbance after 7 mol eq followed by a second rise above 11 mol eq (Fig. 2). The basis for these changes, although unclear, may represent a change from polynuclear cluster binding of Hg(II) to ligation by nonbridging thiolates. A break point was not always obvious with Hg(II) in the fluorescence curves, although maximal protection (about 80%) was usually attained by 7–8 mol eq. The number of Cu(I) equivalents necessary to render the protein resistant was previously reported to be approximately 11, but the transition point was not as obvious as with the Cd(II) titrations (15). The lack of clarity is presumably related to valence instability in the Cu(I) protein. Results with Ag(I) were analogous to those with Cu(I) (Fig. 3). The minimum in the fluorescence curve could be more accurately established to occur near 12 mol eq. The curves shown in Figs. 1–3 are representative data of multiple titrations with each metal. Os(III), Pt(IV), Rh(III), and Ru(III) were also screened in the protease assay at pH 6.5. In each case maximal protection was achieved by 15 mol eq, but transition points were not clear.

**Displacement of Metallothionein-bound Metals—Cd(II) or Zn(II) bound to metallothionein can be displaced by metals that have higher affinities for the protein. Native Cd,Zn-metallothionein and metallothionein separately reconstituted with Ag(I) or Zn(II) were incubated anaerobically with various metals to determine the ability of the added metals to displace the protein-bound cations. After incubation an aliquot of Chelex 100 was added to each tube to remove unbound cations. The samples were centrifuged, and metal analysis was performed on the supernatant (Table III). The Chelex procedure itself did not affect the recovery of Cd,Zn-metallothionein in the supernatant. Twelve mol eq of Bi(III), Cu(I), Hg(II), Pd(II), or Pt(IV) were effective in displacing protein-bound Ag(I). Ag(I), Bi(III), Cu(I), Hg(II), Pd(II), or Pt(IV) displaced most of the metallothionein-bound Cd(II). Incubations with Ag(I), Bi(III), Cd(II), Cu(I), Hg(II), In(III), Pd(II), Pt(IV), or Sb(III) resulted in loss of most of the protein-bound Zn. Only partial Zn release was caused by Os(III), Pb(II), Ru(III), or Sn(II), although concentrations in excess of 12 mol eq of these ions led to total displacement. Co(II), Ir(III), Ni(II), Rh(III), and Tl(III) or (-II1) ions had little to no effect on displacement of either metallothionein-bound Ag, Cd, or Zn.

Several variables in this assay procedure were tested. In a

**Fig. 1. Metal-binding stoichiometry in metallothionein as determined by the proteolytic protection assay.** In these studies 20 μg of apoprotein I were reconstituted anaerobically with increasing mol eq of metal ions. The metal-protein mixture was neutralized to pH 6.0 with HEPES-acetate and subsequently incubated with protease K for 2 h at 37°C. The absorbance at 250 nm (—) and fluorescamine reactivity (— — —) were monitored for each sample. The isolated symbols in the lower right-hand corner of each figure indicate the fluorescamine reactivity of the metalloproteins in the absence of proteolysis.

**Fig. 3. Protection of metallothionein by Ag(I) and Cu(I) toward proteolysis.** The metal-protein mixtures were neutralized to pH 7.8 with HEPES-F and incubated with a 1:30 weight ratio of subtilisin. Similar results were also obtained at pH 6.5 using proteinase K. The assays were as described in the legend to Fig. 1.
study with Ag(I), Bi(III), In(III), and Pb(II) ions the duration of the incubation period was varied from 0.5 to 24 h. No differences were observed in the extent of Cd and Zn displacement from native and Cd,Zn-reconstituted proteins. The effect of pH on metal displacement was investigated. An increase in the extent of Zn displacement from the native protein was observed by decreasing the pH from 6.5 to 5.5 with 10 mol eq of In(III), Os(III), Pt(IV), Ru(III), Sb(III), and Tl(III). The other metals listed in Table II did not exhibit a pH effect. Zn was essentially completely displaced from the protein by 10 mol eq of In and Sb at pH 5.5, only 68 and 22% complete at pH 6.5, respectively, and less than 10% in each case at pH 7.8. This pH effect appears to be predominantly due to the pH-dependent stability of aqueous metal ions. The incubation conditions at pH 5.5 had no apparent effect on the association of Zn with metallothionein. The molar Cd/Zn ratio in native Cd,Zn-metallothionein was unaffected in this Chelex procedure in a pH range from 5.5 to 7.8.

Titrations were carried out using this assay in order to obtain binding stoichiometries. Metals that were effective in the total displacement of Cd or Zn from the protein included Ag(I), Bi(III), Cd(II), Cu(I), Hg(II), In(III), Pd(II), Pt(IV), and Sb(III). Addition of 7 mol eq of Bi(III) or Hg(II) yielded complete release of Cd and Zn from native metallothionein and Zn from the Zn-renatured protein (Fig. 4). Likewise, Cd(II) displaced the Zn ions in Zn-metallothionein in a 1:1 relationship. With increasing Zn displacement, there was a corresponding increase in the Cd content of the protein (Fig. 4). Whereas the protein was depleted of its Zn content by 7 mol eq of In(III) and Sb(III) at pH 5.5, somewhat higher equivalents of Pd(II) and Pt(IV) appeared necessary for total Zn depletion (Fig. 5). Binding studies with Pd(II) and Pt(IV) were complicated by turbidity and occasionally precipitates of the metal-protein complexes. Ag(I) and Cu(I) titrations with metallothionein revealed that 12 mol eq led to complete displacement of protein-bound Zn and maximal concentrations of Ag and Cu in the supernatant (Fig. 6). Cupric ions were also effective in displacing Zn, but the known redox properties of the Cu(II)-metallothionein interaction complicate the interpretation.

Inference of binding stoichiometry using the metal displacement assay was also carried out with Cu_{12} or Ag_{10}-metallothionein as the starting material (Fig. 7). Seven mol eq of Hg(II) totally displaced the protein-bound 12 Cu or Ag ions. There was a corresponding increase in the Hg content of the protein with decreasing Ag or Cu concentrations. The maximal metal content occurred at 7 mol eq.

**Direct Binding Studies—**Apometallothionein reconstituted with 10-14 mol eq Ag(I) was chromatographed by gel filtration. The elution volume containing the protein was quantified for metal and protein concentrations. In 11 separate experiments the protein was recovered with an average Ag content of 11.5 ± 0.7 g atoms/mol protein. This metal content was unaffected if the sample was pretreated with 2 mM β-mercaptoethanol prior to chromatography. The elution position of the Ag-protein was consistent with that of a monomeric protein. Increasing the Ag(I) concentration to 20 mol eq in the incubation mixture resulted in the recovery of molecules with approximately 20 Ag ions bound. Higher molecular weight Ag species were apparent by gel filtration.

Renaturation with a slight excess of Cu(I) led to the isolation of metallothionein containing 10.9 bound Cu ions. However, the yield was quite low (10%) which may be attributed to complications arising from the oxidation lability of the metal-binding thiol groups.
molecule. If 12 mol eq of Cu(I) are allowed to displace Zn from Zn₇-metallothionein prior to chromatography, the protein molecule is recovered in about 30% yield. The Cu content in two experiments was 10.9 and 12.4 g atoms/mol.

Relative in Vitro Metal-binding Affinity—The relative in vitro binding affinities of metals that associate with metallothionein can be ordered by comparing the displacement data in Table III. The proposed ranking of relative affinities is presented in Table IV. The data are not definitive in distinguishing between metals in certain groups such as Hg, Pd, and Pt. The proposed order was substantiated by additional metal displacement studies. Separate incubations of 7 eq of Cd(II) with In(III), Pb(II), and Sb(III)-metallothionein resulted in the apparent displacement of the protein-bound metals. This was inferred by detection of protein-bound Cd in the supernatant with recoveries of 90, 60, and 88%, respectively, of the Cd content of Cd₇-metallothionein. In an analogous experiment, Pb(II)-metallothionein was incubated with 7 mol eq of Zn(II), and no significant quantities of Zn were found in the protein-containing supernatant after the Chelex treatment.

A second approach to ranking in vitro metal-binding affinities was a pH titration of the protein with various metals ligated. As the pH is lowered, metals dissociate from the protein in an order related to the avidity of binding. The displacement of bound metals was monitored by the ultraviolet absorbance of the metalloproteins. Since the extinction in the near ultraviolet is dominated by sulfur → metal change transfer transitions, the pH-dependent loss of absorbance is a reflection of the dissociation of metal ions (37). From the dissociation curves, the pH where the absorbance is attenuated by 50% was determined (Table IV). Since the 2 domains differ in their avidities from metals, the midpoint pH will obviously be an average for the 2 halves. The following midpoint pH values were observed: Cd (3.7), Co (5.8), Cu (2.7), In (4.4), Ni (5.7), Pb (3.8), and Zn (4.8).

**DISCUSSION**

In attempt to determine the possible configurations of metal binding to metallothionein, we have screened numerous metal ions for binding to the apoprotein. Four assays have been employed to identify binding. Chelation was classified as positive if a metal ion: (a) generated a positive ultraviolet absorption difference spectrum comparing binding to the intact apoprotein versus the proteolyzed molecule; (b) protected apometallothionein against proteolysis by subtilisin or proteinase K; (c) displaced Zn or Cd from Cd₇,Zn-metallothionein; and (d) remained associated with the protein after gel filtration of reconstitution mixtures. All assays were not applicable to all the metal ions capable of binding to the protein. Certain metals (Co(II), Ni(II)) did not displace protein-bound Zn(II). Other metal ions, e.g., Hg(II), bound so tenaciously that any stoichiometry may be obtained in the direct binding assay depending on the equivalents added. Ions of at least 18 metals, when present in only a slight excess concentration over available binding sites, satisfied at least 2 of the tests for binding. These metals include Ag(I), Bi(III), Cd(II), Co(II), Cu(I), Hg(II), In(III), Ni(II), Os(III), Pb(II), Pd(II), Pt(IV), Re(V), Rh(III), Ru(III), Sb(III), Tl(III), and Zn(II). In previous studies Ag(I), Au(I), Bi(III), Cd(II), Co(II), Cu(I), Hg(I), Ni(II), and Zn(II) have been documented to associate with metallothionein (9–14).

Experiments with certain metals presented difficulties. Aqueous instability due to disproportionation or hydrolysis, redox reactions of metal ions with the protein, and insolubility with certain anions were among the problems encountered. Therefore, experimental conditions had to be changed to circumvent these difficulties. Whereas In(III) and Sb(III) were prone to hydrolysis near neutrality, buffers at pH 5.5 were acceptable. Although pH 5.5 is higher than pH values where hydrolysis of many of these metal ions normally occurs, complex formation with a ligand as metallothionein will prevent metal hydroxide precipitation (40).

A relative order of in vitro binding affinities determined for several metals correlated well with published affinities of Hg > Cu > Cd > Zn > Ni, Co to metallothionein (9, 41). Contrary to this order, Waalkes et al. (42) recently reported the following order based on Zn displacement studies from Zn₇-metallothionein: Cd > Pb > Cu > Hg > Zn > Ag > Ni > Co. Boulanger et al. (7) proposed an order of affinity in the β domain to be Cu > Zn > Cd. Data presented in this study clearly makes these latter schemes questionable. The order proposed in Table IV agrees well with established association constants for metals and cysteine (Hg > Cu(I) > Ag > Pb > Cd > Ni > Zn > Co) and with sulfides (Hg > Ag > Pb > Cd > Zn) (40, 43–44). The pH displacement results with metallothionein suggest that Cd and Pb bind with the similar affinities, whereas the Zn displacement data predicts greater avidity in the Cd binding. Contrary to binding studies with cysteine, Zn associated to metallothionein more tenaciously than did Ni.

Binding stoichiometries of these various metals were assessed by monitoring the equivalencies yielding complete protection against proteolysis and total displacement of Zn or Cd ions from Cd₇,Zn-metallothionein. The basis of the proteolytic protection assay was verified with Cd(II), Co(II), Ni(II), and Zn(II) where maximal binding is known to be 7 ions/molecule (5, 9). The Zn displacement assay is valid only for metals that possess a substantially greater affinity for the protein than does Zn(II). Other metals that were found to exhibit saturation binding at 7 mol eq include Bi(III), Hg(II), In(III), Pb(II), Sb(III), and probably Pd(II). Vasak et al. (8) and Bernhard et al. (10) reported tetrahedral binding of Bi(III), Hg(II), and Pb(II) from spectral studies of the metal-reconstituted proteins, but titrations were not performed to verify binding stoichiometries. Others have observed metallothionein binding of Bi and Hg in metal-injected animals, but the bound Bi and Hg contents were low (45, 46).

Metals such as Au(III), Re(V), Rh(III), Pt(IV), Ru(III), Os(III), and Tl(III) give positive indication of binding, but data on stoichiometries were unclear. The ambiguity with these ions is probably related to aqueous instability of the ions or unstable complexes with the protein. Experiments with Au(III) revealed a break point of about 12 in both the proteolytic protection assay and Zn displacement from Zn₇-metallothionein. Although the transition point seems clear, the nature of Au(III) as a potent oxidant makes interpretation of these data unclear. Other redox-active metal ions would also confuse data interpretation. Direct binding studies were carried out with In(III), Pd(II), Pt(IV), Rh(III), and Sb(III). Whereas In and Sb exhibited saturation binding between 5.5 to 7 metal ions/protein molecule with good recovery of protein, recovery of protein renatured with Rh, Pd, and Pt was quite low, thereby obscuring an interpretation.

Cu(I) and Ag(I) appear to bind in clusters as M₇₇-metallothionein. In the proteolytic protection assay 12 mol eq of Ag(I) protects the protein against proteolysis. Approximately 12 mol eq of Ag(I) or Cu(I) are necessary for the complete displacement of Zn from Zn₇-metallothionein. In direct binding studies, renaturation of the apoprotein with a slight excess of Ag(I) followed by gel filtration resulted in the recovery of Ag-metallothionein with an average Ag content of 11.5 g atoms of Ag/mol of protein. A comparable number is obtained
in the molecule resulting from Cu(I) displacement of Zn from the Zn7-protein. Thus, a binding stoichiometry for Ag and Cu of 12 eq is apparent from both constitution and Zn-displacement studies. Addition of Ag(I) is excess of 20 mol eq results in collapse of the cluster structures to yield a metal cysteinyl ratio of about 1. At lower equivalents Ag(I) appears to bind in polynuclear clusters analogous to Cu(I). We have preliminary data that Ag(I) binds cooperatively and initially in the β domain as does Cu(I). Ag-metallothionein appears to represent an air-stable model for the M12-protein complex.

Metallothionein binds metal ion in 2 distinct configurations. The distribution of metals in the M1 state is M6α and M6β, and the metals are coordinated tetrahedrally (3, 5). The 12 Cu(I) ions are bound in polynuclear clusters with a distribution of M6α and M6β. From the cysteine content in the domains, the β domain metal:ligand stoichiometry is Cys9Cu6Cys9. In order for the Cu ions to have quasi-equivalent bonds, the Cu(I) ions should be coordinated trigonally. One possible trigonal orientation is depicted in Fig. 9. In this complex, all thiocianates are bridging sulfurs. In known structures of several small Cu-thiolate complexes, the usual coordination chemistry is trigonal Cu(I) ions and bridging sulfurs (19-22). Ag(I) forms a M5S7 complex that is similar in structure and coordination stereochemistry to the CuS7 complex (22). Two distinct binding configurations have also been reported in another system. Beltramini et al. (47) recently reported that whereas the Neurospora 25-residue metallothionein polypeptide normally binds 6 Cu(I) ions/molecule, the Cd(II), Hg(II), and Zn(II) renatured molecules exhibit a metal to protein stoichiometry of 3. The two states in metallothionein are probably interconvertible. The Zn7-protein can be converted to the M12 state by metal displacement with 12 mol eq of Cu(I) or Ag(I). Conversely, the M12-protein can be reverted to the M7 state by displacement with 7 mol eq of Cu(Hg). The higher binding content of Cu-metallothionein compared to the Zn-form may result in 2 different tertiary structures. If metallothionein functions in distinct processes for Zn and Cu, a conformational difference between the M7- and M12-proteins would permit the cell to discriminate between the 2 forms. In support of distinct structures, the Stokes radius of Zn-metallothionein is slightly greater than that of the Cu-protein. Resolution of Cu- and Zn-proteins has also been observed by Brenner and colleagues (48, 49).

In many cells metallothionein appears to exist with varying ratios of bound Zn and Cu. It is unclear whether the protein exists in most cells as a mixed Cu,Zn-protein or whether pure Zn or Cu states of the molecule occur. The 2 domains in the protein appear to be relatively independent of each other. However, as the interdomain-connecting peptide Ly82Ly83 is short, the clusters may be spatially close. The stoichiometry of a given domain may be influenced by the tertiary conformation of the other domain. Prior formation of cluster A by Zn could impart constraints in the folding of the β domain, thereby precluding formation of a Zn-type tetrahedral center mixed with a Cu-type trigonal center. Answers to these questions will have an important bearing on the yet unresolved function of metallothionein. Further studies to elucidate the cluster structure of Cu-metallothionein and its coordination geometry are in progress.

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FIG. 9. Proposed coordination complexes for the β domain of metallothionein. In the M7 and M12 states of the protein, the β domain appears to coordinate 3 and 6 metals, respectively, via 9 cysteinyl residues.
Metal Binding in Metallothionein

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THE PROTEOLYTIC PROTECTION ASSAY

To determine the effect of metal ions on the activity of the proteolysis assay, the metal ions were tested for their interference in the proteolysis of a 5% casein and caseinase under conditions similar to those described. The metals were tested for their capacity to inhibit the proteolysis of casein and to selectively inhibit the proteolysis of caseinase. The assay was performed in triplicate, and the results were expressed as the percentage of the control. The effect of metal ions on the activity of the proteolysis assay was determined by comparing the activity of the proteolysis assay in the presence and absence of the metal ions.

TABLE 1

| Metal | % Protection | Extinction of Protein |
|-------|--------------|----------------------|
| Ag(I) | 12           | 5.3 ± 0.5            |
| A(II) | 30           | 7.8 ± 1.2            |
| Cd(II) | 10           | 5.5 ± 0.3            |
| Zn(II) | 30           | 7.8 ± 1.2            |
| Cu(II) | 10           | 5.5 ± 0.3            |
| Cd(II) | 30           | 7.8 ± 1.2            |
| Cu(II) | 30           | 7.8 ± 1.2            |
| Zn(II) | 30           | 7.8 ± 1.2            |
| Cu(II) | 30           | 7.8 ± 1.2            |
| Zn(II) | 30           | 7.8 ± 1.2            |
| Cu(II) | 30           | 7.8 ± 1.2            |
| Zn(II) | 30           | 7.8 ± 1.2            |
| Cu(II) | 30           | 7.8 ± 1.2            |

The data in Table 1 show that the proteolysis assay is sensitive to the presence of metal ions. The inhibition of the proteolysis assay by metal ions is dependent on the metal ion and the concentration of the metal ion used. The inhibition of the proteolysis assay by metal ions is not specific to a single metal ion, and the inhibition is observed with all metal ions tested, including Ag(I), Cd(II), Zn(II), and Cu(II). The inhibition of the proteolysis assay by metal ions is also dependent on the concentration of the metal ion used. The inhibition of the proteolysis assay by metal ions increases with increasing concentration of the metal ion used. The inhibition of the proteolysis assay by metal ions is also dependent on the time of incubation of the metal ions with the proteolysis assay. The inhibition of the proteolysis assay by metal ions decreases with increasing time of incubation of the metal ions with the proteolysis assay.

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TABLE III

Metallography Displacement of Metallofthinin - Basic Metals

Methylamine (50 μg) in HEPES-urate solution was incubated with various metals as described in Methods to determine the ability of these metal ions to displace protein-bound metal. The native GSH-protein (1) was incubated with MeOH and then with various metals at pH 7.5. The metallothionein (2) was incubated with metal proteins at pH 7.5. The supernatant metal concentration was measured with Ag nitrate. The supernatant was then analyzed by a Chelex 100 slurry as described in Table I. Metal analysis was performed on the supernatant after centrifugation.

| Displacing Metal | Cds-Protein | Ag-Protein |
|------------------|-------------|------------|
| None             | 3.7 ± 1.0   | 2.8 ± 0.6  |
| Cu(I)            | 0.0 ± 0.1   | 0.0 ± 0.0  |
| Zn(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Cd(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Mg(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Fe(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Mn(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Ni(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Co(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Cu(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Cd(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Zn(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Mg(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Fe(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Mn(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Ni(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Cu(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Cd(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Zn(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Mg(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Fe(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Mn(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Ni(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Cu(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Cd(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Zn(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Mg(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Fe(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Mn(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Ni(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Cu(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Cd(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Zn(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Mg(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Fe(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Mn(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Ni(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Cu(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Cd(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Zn(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Mg(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Fe(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Mn(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Ni(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Cu(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Cd(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Zn(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
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| Fe(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Mn(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |
| Ni(II)           | 0.0 ± 0.0   | 0.0 ± 0.0  |