

Formation of the Metal-Thiolate Clusters of Rat Liver Metallothionein

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The isoforms of rat liver apo-metallothionein (MT) were reconstituted *in vitro* with Cd and Zn ions to study the order of binding of the seven metal sites. Reconstitution with seven Cd ions resulted in a metalloprotein similar to induced Cd,Zn-MT by the criteria of electrophoretic mobility, insensitivity to proteolysis by subtilisin and the pH-dependent release of Cd. Proteolytic digestion of MT reconstituted with sub-optimal quantities of Cd followed by separation of Cd-containing polypeptide fragments by electrophoresis and chromatography revealed metal ion binding initially occurs in cluster A. Upon saturation of the four sites in cluster A, binding occurs in the three metal center, cluster B. Samples reconstituted with one to four Cd or Zn ions per protein molecule, followed by digestion with subtilisin, yielded increasing amounts of a proteolytically stable polypeptide fragment identical with the α fragment domain encompassing the four metal center. Samples renatured with five to seven Cd ions per MT molecule showed decreasing quantities of α fragment and increasing amounts of natively like MT. The binding process in each domain is cooperative. Reconstitution of apo-MT with two Cd ions followed by proteolysis yields a 50% recovery of saturated Cd₄- α cluster. Likewise, when Cd₅-renatured MT was digested with subtilisin, 30% of the molecules were identified as Cd₇-MT with the remainder as Cd₄- α fragment.

Metallothionein (MT) induced by Cd or Zn ions binds seven metal ions per molecule, and this chelation involves the cysteines in the ligand field. The metal ions are bound in two separate polynuclear metal-cysteine clusters (1-3). One cluster (cluster A) contains four metal ions bound to eleven cysteines, five of which exist as thiolate bridges connecting adjacent metal ions. The other center (cluster B) binds three metal ions through nine cysteines and three thiolate bridges (1,2). We demonstrated that the two centers are enfolded by separate regions of the 61 amino acid polypeptide chain (4,5). The three-metal center domain comprises the NH₂-terminal half of the molecule and the four-metal domain is formed by the COOH-terminal half. The two clusters appear to differ in their metal-binding properties. Results from ¹¹³Cd-NMR experiments and our isolation of a peptide fragment of MT containing the four-metal center reveal that the metal ions in Cd,Zn-MT are arranged such that cluster A contains four Cd ions and cluster B contains on the average two Zn ions and one Cd ion (1,4). Calf liver MT was found to contain three Cu ions selectively

in cluster B with Cd displaceable Zn ions located in cluster A (6). These distribution differences may result from the two clusters having similar but not identical tertiary structures which may alter the coordination preferences. This suggestion is consistent with ¹H-NMR data (7). Since a predominant physiological form of MT is Zn-MT, we were interested in determining the order of metal binding to the clusters by utilizing *in vitro* metal ion reconstitution with Cd and Zn.

It is well known that MT is capable of being reversibly reconstituted. Kagi and Vallee (8) and Pulido et al. (9) demonstrated that the metal ions could be removed from MT at pH 2 or with EDTA with the concomitant loss of absorbance at 250 nm. Addition of Cd²⁺ to apo-MT at neutral pH restored the ultraviolet absorption. In UV and CD spectroscopic studies, Law and Stillman (10) showed that renaturation of apo-MT resulted in a natively like protein, although slight differences from the native form were observed. Recently, Vasak and Kagi (11) reported that the binding of Co²⁺ to thionein occurs in metal-thiolate clusters analogous to the binding of Cd²⁺ by native Cd,Zn-MT.

In our studies, apo-thionein was prepared by acidification in 0.1 N HCl followed by gel filtration in 0.01 N HCl. The metal-free protein was

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identified in the column effluent by monitoring the fluorescamine reactivity and quantified by amino acid analysis. To achieve reconstitution, metal ions were added to thionein in 0.01 N HCl in a N_2 -purged glove bag. The solution was neutralized to pH 7.5 by the addition of potassium phosphate. All solutions were deaerated and bubbled with N_2 prior to use in the anaerobic glove bag.

In order to study the mode of cluster formation in MT by *in vitro* reconstitution, it was necessary to initially demonstrate that under our conditions natively like properties could be restored in metal reconstituted apo-metallothionein. The properties selected as criteria of binding integrity included ultraviolet absorption, pH-dependent displacement of bound metal ions and susceptibility to proteolysis. By these criteria, anaerobic addition of seven Cd ions to thionein results in a metal-containing protein with characteristics of native Cd,Zn-MT. The absorption at 250 nm of reconstituted Cd-MT was similar to that of native Cd,Zn-MT in the absorbance per Cd ion. The pH-dependent displacement of Cd ions was identical in the two samples, suggesting that similar mercaptide chelation exists for each. Native Cd,Zn-MT is completely resistant to proteolysis by subtilisin even when incubated at high concentrations of the protease for prolonged times at elevated temperatures. Apo-MT, on the other hand, is digested to small peptides under similar conditions. Both isoforms of apo-MT reconstituted with seven Cd ions are resistant to proteolytic degradation. The metal reconstituted MT isoforms also exhibit the same electrophoretic mobility as native MT isoforms. These results suggest that the reconstitution conditions are adequate for restoration of natively like binding of Cd ions.

Apo-MT was renatured under the same conditions but with less than saturating amounts of Cd to determine the order of filling of the binding sites. The reconstituted samples showed increasing ultraviolet absorbance as a function of added metal ion and the absorbance rise per Cd equivalent was similar for each sample. Addition of Cd in concentrations exceeding saturation of the seven metal sites (e.g., eight Cd ions per molecule) did not increase the absorbance beyond the value obtained for seven Cd ions. The absorption properties of reconstituted samples were stable to proteolysis by subtilisin. However, apo-MT preincubated with the protease prior to metal ion reconstitution showed an absorption spectrum that more closely resembled apo-MT than the protein incubated subsequent to reconstitution, indicating that the Cd-thiolate charge transfer transi-

tions seen in MT require the intact polypeptide.

Since the binding of Cd, even at suboptimal levels, imparts a resistance to proteolytic digestion, proteolysis can be utilized to probe the location on the polypeptide chain where binding initiates. The binding sites on MT are enfolded in two distinct domains which we demonstrated could be separated by proteolysis (4). Metal binding initially within one cluster should presumably confer proteolytic resistance to that domain, whereas the unoccupied domain would be digested. If binding occurred randomly along the polypeptide chain, treatment of the samples with subtilisin

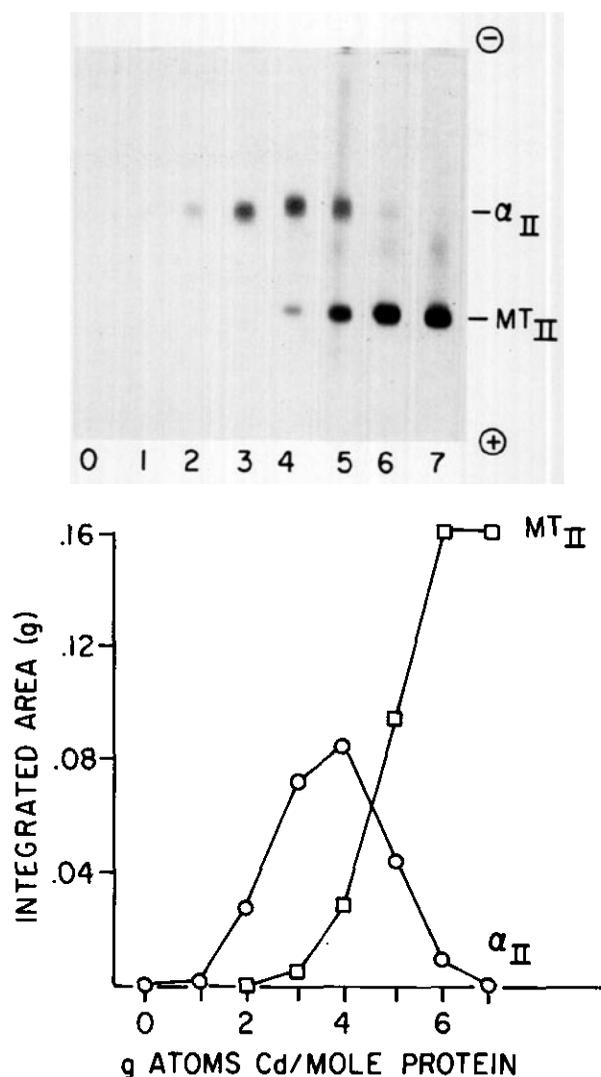


FIGURE 1. Integration of Coomassie-stained protein bands after polyacrylamide gel electrophoresis of apo-MT II reconstituted with 0 to 7 Cd ions. The samples (20 μ g per gel) were pretreated with subtilisin prior to electrophoresis. The stained gels were scanned, and the areas under the peaks were quantified by weighing the cut out peaks.

would generate a differing peptide pattern as increasing amounts of Cd were added.

Samples of apo-MT reconstituted with Cd were incubated with subtilisin prior to nondenaturing gel electrophoresis to resolve Cd-containing peptides. In the reconstitution of apo-MT II with low levels of Cd (one to four Cd ions/MT molecule), a prominent Coomassie blue stained band was observed which had a mobility identical with that of the COOH-terminal α domain of MT II (α fragment) (Fig. 1). Integration of the densitometric scans of the gels showed that the quantity of the α fragment increased in samples binding up to four Cd ions per molecule and subsequently decreased concomitantly with an increase in a stained band corresponding to native MT II. These data suggest that binding is ordered and occurs initially in cluster A followed by cluster B. This conclusion is supported by an evaluation of the peptides generated by the incubation with subtilisin which revealed a pattern that was qualitatively similar in each reconstituted sample, but the concentration of the peptide mixture decreased as the number of Cd ions bound increased. In order to verify that the subtilisin resistant peptide was the authentic α fragment, the experiment was repeated with isoform I of MT, since the α I fragment exhibits a different electrophoretic mobility than α II. Samples reconstituted with up to four Cd or Zn ions followed by incubation with subtilisin revealed an increasing quantity of a peptide fragment identical in mobility to that of α I. Binding of Cd of Zn in excess of four ions per molecule showed a decreasing amount of α I and a concomitant increase in native MT I.

To confirm that the proteolytically resistant peptide observed in samples with one to four metals ions bound was the α fragment, samples were reconstituted with four Cd, digested with subtilisin and then chromatographed on Sephadex G-75. The only major peptide eluted in the column effluent with a K_d of 0.7, a value identical to that of the Cd₄- α domain. The elution profile of Cd was coincident with the peptide absorbance at 230 nm. Cd₆-reconstituted MT eluted with a K_d of 0.54, a value identical to that of native Cd₄Zn-MT. Amino acid analysis of the Cd-containing peptide prepared from subtilisin-treated Cd₄-reconstituted apo-MT I revealed a composition similar to that seen with the α fragment. The metal content of the peptide averaged 3.8 Cd per molecule.

Reconstitution experiments were also done with purified α I fragment which had been demetalized. Apo- α I was reconstituted with 0 to 4 Cd equivalents per mole protein followed by incubation with subtilisin. An aliquot was taken for

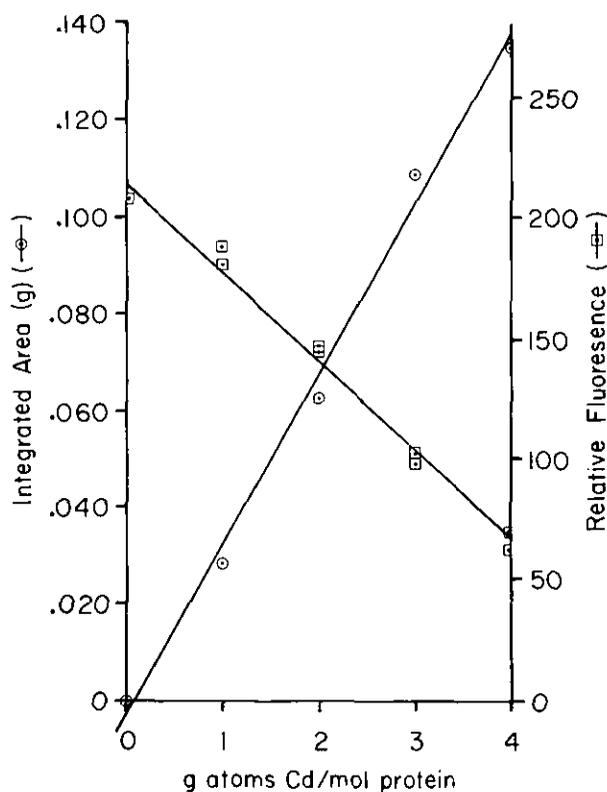


FIGURE 2. Integration of Coomassie-stained apo- α I after reconstitution with 0 to 4 Cd ions per molecule and electrophoresis on 7.5% polyacrylamide gels. The integration of peak areas from 10 μ g of sample (-o-) was performed as described in the legend to Figure 1. In addition, 20 μ g of each sample was reacted with 0.1 mg of fluorescamine in 0.2 M sodium borate, pH 8.5, and the fluorescence was determined (-□-). The lines were determined by linear regression analysis.

nondenaturing gel electrophoresis, and recovery was assessed by integration of the densitometric scans of the gels. As can be seen in Figure 2, there was a linear relationship between the concentration of the α fragment and the number of Cd ions bound. The reactivity of the samples with fluorescamine was monitored by fluorescence for two separate experiments (Fig. 2). The fluorescence observed correlates with the number of primary amino groups and therefore reflects the proteolytic susceptibility of the samples. The fluorescence yield is related inversely to the number of Cd ions bound. The linearity of these plots would be consistent with cooperative binding of metal ions to the clusters of MT.

If cooperative binding occurs, the initial phase should be a cooperative saturation of cluster A. For example, reconstitution with two Cd ions followed by proteolysis should result in 50% of the molecules being recovered as Cd₄- α . Figure 3A

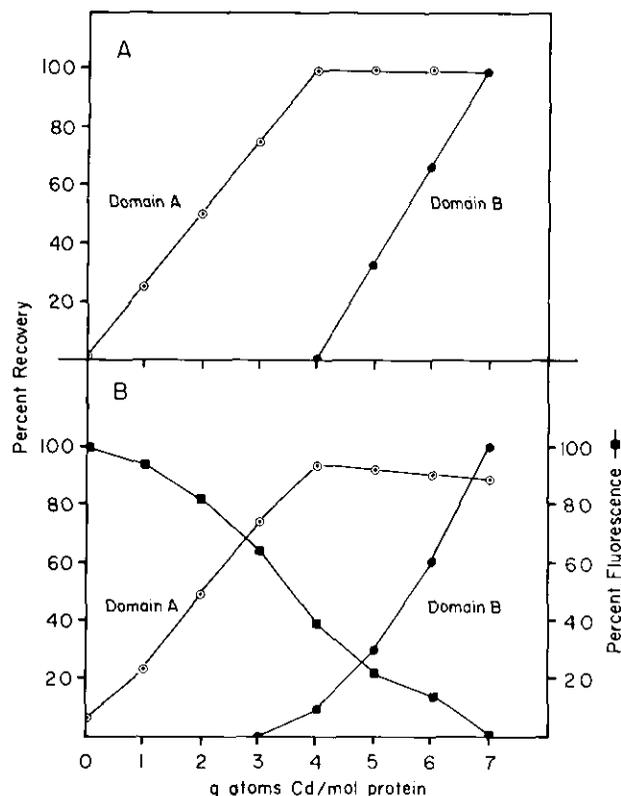


FIGURE 3. Plots of (A) theoretical curve for positive cooperativity in the ordered binding of Cd to the two clusters of MT and (B) the actual recoveries of metal-cluster domains A and B after treatment with subtilisin and gel filtration on Sephadex G-25. The recoveries of protein in the column excluded volume were quantified as described in the text. The fluorescamine reactivity of the pooled internal volume fraction from each sample was determined (■). The value for apo-MT was set as the 100% value.

shows the theoretical curves for positive cooperativity in the metal ion binding to the two domains. The two curves represent the recoveries of saturated four-metal and three-metal domains. The actual recoveries of the two domains (Fig. 3B) were determined from the amino acid compositions of the reconstituted samples exposed to proteolysis and subsequently gel filtered to separate MT and α fragment from digested peptides. The domain of MT I containing cluster A contains all the Ala and Val residues in the MT molecule. Quantitation of these residues equates with the recovery of this domain. Quantitation of the cluster B containing domain was based on recovery of methionine in that the only Met residue is NH₂-terminal. The recoveries of the two domains are similar to the theoretical values for positive cooperativity in binding. Also shown in Figure 3B is the fluorescamine reactivity curve of the Cd-reconstituted samples. To ensure against only

Table 1. Metal content of reconstituted sample.

| Cd ²⁺ added/Apo-MT | Sephadex G-75 separated molecules, g-atoms Cd/mole | |
|-------------------------------|--|----------|
| | MT | α |
| 7 | 6.8 | — |
| 5 | 6.7 | 4.1 |
| 4 | 6.5 | 3.8 |
| 2 | — | 4.0 |

partial digestion, 16 hr was allowed for proteolysis. The time observed for completion of digestion of apo-MT, Cd₂- and Cd₄-reconstituted MT was about 2 hr.

Quantitation of the metal content of various proteolyzed reconstituted samples, after separation of MT and α fragment by Sephadex G-75 column chromatography, confirmed that cooperative binding had occurred (Table 1). The α fragment recovered from Sephadex G-75 after reconstitution of apo-MT with only 2 Cd ions per mole α . The Cd₄-reconstituted MT was resolved into a major α fraction (3.8 Cd/molecule) and a minor MT peak containing 6.5 Cd per molecule. Likewise, Cd₅-reconstituted MT was resolved into a population of Cd-saturated MT molecules and a population of metal-saturated α fragments.

The binding of Cd and Zn ions by metallothionein is an ordered process with cluster A exhibiting a greater binding affinity than cluster B. One intermediate (Cd₄-MT) can be isolated, but other intermediate products are not observed due to cooperative binding within each domain. Cooperativity is evident within each domain, but not between domains.

In a recent publication, Vasak and Kagi (11) carried out *in vitro* Co (II) reconstitution of rabbit liver MT-I to study the metal cysteine clusters. Their electronic and EPR spectroscopic data not only support the existence of two metal clusters in MT, but also suggest that the mode of formation of the cluster was stepwise. The first four metal ions bound produced virtually no magnetic interaction and therefore were presumably distributed between the two clusters. This is in marked contrast to Cd and Zn binding. The remaining three Co ions gave increasing interaction as the separate sites became linked, yielding Co₇-MT which is diamagnetic presumably due to antiferromagnetic coupling. Vasak and Kagi suggested that the first four metal ions produced a steric configuration necessary for binding of the remaining three (11). The formation of the metalthiolate clusters in rat liver MT with Cd or Zn ions is clearly ordered, and the cooperative bind-

ing should yield interaction as cluster A is formed. The difference observed between reconstitution with Co and Cd or Zn is intriguing. The two domains may have unique structures that result in different coordination preferences. Cd and Zn show a preference for cluster A and their binding is cooperative. We have preliminary data that Cu binding also occurs in an ordered process, but that saturation of cluster B is the initial phase. It is possible that no cluster preference exists for Co binding. The different metal binding properties of the two domains may be significant in the function of MT.

The cooperative binding of Cd and Zn implies that the unloading process may also be cooperative. In Zn-MT the order presumably would be release of the metal ions in cluster B prior to those in cluster A. The simultaneous release of three or four metal ions may be an important feature in the still unresolved function of MT.

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