Order of Metal Binding in Metallothionein*

(Received for publication, May 2, 1983)

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Purified isoforms of rat liver apometallothionein were reconstituted in vitro with Cd and Zn ions to study the order of binding of the 7 metal sites in the two separate metal clusters, one containing four metal ions (cluster A) and the other containing three (cluster B). Reconstitution with 7 Cd ions resulted in a metallothionein similar to induced Cd,Zn-metallothionein by the criteria of electrophoretic mobility, insensitivity to proteolysis by subtilisin, and the pH-dependent release of Cd. Proteolytic digestion of metallothionein reconstituted with suboptimal quantities of Cd followed by separation of Cd-containing polypeptide fragments by electrophoresis and chromatography revealed metal ion binding initially occurs in the 4-metal center, cluster A. Upon saturation of the 4 sites in cluster A, binding occurs in the 3-metal center, cluster B. Samples reconstituted with 1 to 4 Cd ions per protein molecule, followed by digestion with subtilisin, yielded increasing amounts of a proteolytically stable polypeptide fragment identical with the α fragment domain that is known to encompass the 4-metal center. Samples reconstituted with 5 to 7 Cd ions per metallothionein molecule showed decreasing quantities of α fragment and increasing amounts of native-like metallothionein. Similar results were obtained in reconstitution studies with Zn ions. Samples reconstituted with 7 Cd eq followed by incubation with EDTA revealed that cluster B Cd ions were removed initially. The binding process in each domain is cooperative. Reconstitution of apometallothionein with 2 Cd ions per protein followed by proteolysis yields a 50% recovery of saturated Cd, α cluster. Likewise, when Cd-reconstituted metallothionein was digested with subtilisin, 30% of the molecules were identified as Cd-metallothionein with the remainder as Cd, α fragment.

Metallothionein is a low molecular weight cysteine-rich protein found in all eukaryotic cells and is capable of binding a variety of metal ions (1–4). Two polymorphic forms of the protein exist that differ in primary structure (5–9). The isoforms are inducible in most cells by a variety of agents including metal ions and dexamethasone through transcriptional regulation (1, 2, 10–12). Amino acid sequences of metallothioneins from a variety of species show extensive homologies and an invariance in positions of the 20 cysteines (1, 13). Metallothionein induced by Cd or Zn ions bind 7 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 (14, 15). One cluster (cluster A) contains 4 metal ions bound to 11 cysteines, 5 of which exist as thiolate bridges connecting adjacent metal ions. The other center (cluster B) binds 3 metal ions through 9 cysteines and 3 thiolate bridges (14, 15). The 2 centers are enfolded by separate regions of the 61-amino acid polypeptide chain (16, 17). The 3-metal domain consists of the NH2-terminal half of the molecule and the 4-metal domain is formed by the COOH-terminal half. Although diffracting crystals of rat liver Cd,Zn metallothionein II have been prepared (18), the three-dimensional structure of metallothionein has not yet been determined. A model for the structure has been proposed based on 113Cd and 1H-NMR data (19). The model predicts that the polypeptide chain is tightly wrapped around the metal centers and the clusters are stabilized by H-bonding between side chain hydroxy residues and the peptide backbone.

The 2 clusters appear to differ in their metal-binding properties. Results from 113Cd-NMR experiments (14) and our isolation of a peptide fragment of metallothionein containing the 4-metal center (16) reveal that the metal ions in Cd-induced mammalian Cd,Zn metallothioneins are arranged such that cluster A contains 4 Cd ions and cluster B contains the average 2 Zn ions and 1 Cd ion. Calf liver metallothionein was found to contain 3 Cu ions selectively in cluster B with Cd displacable Zn ions located in cluster A (20). In the present study we demonstrated the order of binding to the metal clusters of metallothionein using a single type of metal ion. This was done since a predominant form of naturally occurring metallothionein in human liver and uninduced rat liver is Zn-metallothionein. Our results demonstrate that cluster A fills initially in a cooperative fashion followed by cooperative binding to cluster B.

MATERIALS AND METHODS

Supplies and reagents were obtained as previously described (16). Male Sprague-Dawley rats of approximately 250 g were purchased from Simmerson and were fed commerical rat chow ad libitum. Metallothionein was induced by administering 6 subcutaneous injections of CdCl2 (2.5 mg of Cd/kg body weight) to the rats on alternate days. The purification of the protein from livers excised 1 to 5 days following the last injection was a modification of a procedure previously described (7, 21). The purification protocol involved heating a 30% homogoneous to 60 °C followed by a chloroform-ethanol extraction of the clarified supernatant. The metallothionein in the ethanol phase was precipitated with 2 volumes of acetone, resuspended in 10 mM Tris-Cl, pH 8.5, and applied to Sephadex G-75 equilibrated and eluted in the same buffer. The metallothionein-containing fraction was directly applied to a DEAE-cellulose column (2.2 × 10 cm) equilibrated in the same buffer. A 1-liter salt gradient of 0 to 30 mM NaCl was used to elute the protein. The 2 isoforms were concentrated by lyophilization, desalted on Sephadex G-25 in 10 mM Tris-Cl, pH 8.5, and separately rechromatographed on a second DEAE-cellulose column following lyophilization, the samples were desalted on Sephadex G-25 equilibrated and eluted with 10 mM potassium phosphate, pH 7.8. Samples were stored at ~70 °C in the dilute phosphate buffer.

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*This work was supported by National Institutes of Health Grant GM 30482. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The designated terms used are clusters A and B, the 4- and 3-metal centers, respectively; α, the COOH-terminal domain of metallothionein which encompasses cluster A.
Under these conditions, approximately 20 mg of each isoform of Cd,Zn metallothionein can be recovered in purified form from 10 rat livers. The purity of the isoforms was assessed by non-denaturing polyacrylamide gel electrophoresis, ultraviolet absorption spectroscopy, and amino acid analysis. The purified proteins have a 250 nm/280 nm absorption ratio exceeding 20 due to the Cd-cysteine coordination complex and the absence of aromatic amino acids. The amino acid compositions are known for the isoforms, and purity is readily apparent as the proteins are devoid of His, Arg, Phe, Tyr, Trp, and Leu.

Apometallothionein was prepared by acidification of the native protein in 0.1 N HCl followed by gel filtration chromatography in 0.01 N HCl. The metal-free protein was identified in the column effluent either by monitoring for absorbance at 215 nm or by fluorescamine reactivity quantifying primary amino groups. The metal content of the resulting apoprotein is less than 0.1 µg atom of metal ions per mg of protein.

To achieve reconstitution, metal ions were added to metal-free thionein in 0.01 N HCl enclosed in a N₂-purged glove bag at 25 °C. Appropriate metal concentrations were added to the anaerobic bag. In certain reconstitution experiments, subtilisin (protease:metallothionein, 1:30 by weight) was added to the anaerobic bag. In all solutions, the anaerobic bag was gassed with N₂ prior to use in the anaerobic bag. In certain reconstitution experiments, subtilisin (protease:metallothionein, 1:30 by weight) was added to the anaerobic samples and digestion was allowed to proceed at 25 °C for 18 h in N₂-purged sealed tubes.

Amino acid analysis performed on a Beckman 120C analyzer was used to assess purity and to quantify protein concentrations. Metal analysis was carried out on a Perkin-Elmer model 350A spectrometer. Absorption spectra were recorded on a Cary 118 spectrometer. Fluorescence measurements were made on a Perkin-Elmer 650-10S spectrophotofluorometer of samples treated with fluorescamine (22). Polyacrylamide gel electrophoresis was performed at pH 8.9 in non-denaturing 7.5% gels (23). Cylindrical gels were scanned at 500 nm after staining with Coomassie blue R-250. Proteolytic peptides were resolved by ascending chromatography on Brinkmann thin layer cellulose plates (20 cm). The solvent system was a mixture of pyridine/butanol/acetic acid/water (10:15:3:12, by volume). Peptides were identified by spraying the plates with 0.2% ninhydrin in 95% aqueous acetone.

RESULTS

In order to study the metal binding order in metallothionein by in vitro reconstitution, it was necessary to initially demonstrate that the properties of metal reconstituted apometallothionein are similar to those of the native protein. 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 7 Cd ions to thionein results in a metal-containing protein with characteristics of native Cd,Zn metallothionein. Fig. 1 shows the ultraviolet absorption spectra of Cd-reconstituted metallothionein, apometallothionein, and native metallothionein. The absorption at 250 nm of reconstituted Cd metallothionein exceeds that of native Cd,Zn metallothionein. The absorption results primarily from Cd-sulfur charge transfer transitions (24, 25) and, therefore, reflects the Cd content of the sample. Cd,Zn metallothionein isolated from Cd-induced rat livers typically averages 5 Cd and 3 Zn ions per molecule. The absorbance per Cd ion in the two samples is comparable at 250 nm. Since the extinction at 250 nm is proportional to the bound Cd content (24, 25), the absorbance can be used as an indicator of the content of bound metal ion. The absorbance at 250 nm as a function of pH for Cd⁻ and Cd₃⁻ reconstituted metallothionein and native Cd,Zn metallothionein is shown in Fig. 2. The pH-dependent displacement of Cd ions is similar suggesting that similar mercaptide chelation exists for each.

Native Cd,Zn metallothionein is completely resistant to proteolysis by subtilisin even when incubated with high concentrations of the protease for prolonged times at 37 °C.

![Fig. 1. Ultraviolet absorption spectra of isoform I of apometallothionein (A), native Cd,Zn metallothionein (B) and Cd₇-reconstituted metallothionein (C). The samples contained 50 µg of protein per ml of 30 mM potassium phosphate, pH 7.8.](image)

![Fig. 2. Hydrogen ion displacement of metallothionein-bound Cd in native metallothionein (Δ), Cd₇-reconstituted metallothionein (O), and Cd₇-reconstituted metallothionein (Φ). The absorbance at 254 nm was monitored as a function of pH. The absorbance at pH 8 was used as the 100% bound value. Each sample was 50 µg of protein per ml and titrated with 0.1 N HCl.](image)
Order of Metal Binding in Metallothionein

The absorbance at 250 nm per Cd ion added was similar for each sample. Addition of Cd in concentrations exceeding saturation of the 7 metal sites (e.g. Cd ions per molecule) does not increase the absorbance beyond the value obtained for 7 Cd ions. Incubation of reconstituted samples with subtilisin for 16 h at 25 °C did not affect the absorption properties of the samples (Fig. 4, inset). In order to determine whether proteolysis affects Cd binding, apometallothionein was incubated with subtilisin before and after reconstitution with 7 Cd ions. Apometallothionein preincubated with the protease prior to metal ion reconstitution showed an absorption spectrum that more closely resembled apometallothionein than the spectrum obtained when the protein was incubated with the protease after renaturation.

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 metallothionein are enfolded in 2 distinct domains which can be separated by proteolysis (16). 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 would generate a differing peptide pattern as increasing amounts of Cd were added.

Samples of apometallothionein reconstituted with Cd were incubated with subtilisin prior to nondenaturing polyacrylamide gel electrophoresis in order to resolve Cd-containing

Fig. 3. Nondenaturing polyacrylamide gel electrophoresis of proteolyzed native Cd,Zn metallothionein isoforms I and II (gels A), Cd-reconstituted metallothionein (MT) (gels B), and apometallothionein isoforms (gels C). The samples were preincubated with subtilisin (1:30 weight ratio of protease to metallothionein) for 16 h at 25 °C prior to loading 20 µg per gel. The anode is at the gel bottom. The mobilities of the reconstituted metallothionein are the same as those of the native metallothionein isoforms.

Fig. 5. Integration of Coomassie-stained protein bands after polyacrylamide gel electrophoresis of proteolyzed apometallothionein II reconstituted with 0 to 7 Cd ions. The samples (20 µg 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. No correction was made for different staining abilities of α and metallothionein (MT).
peptides. When apometallothionein II was renatured with low levels of Cd (1 to 4 Cd ions/metallothionein molecule), a prominent band was observed which had a mobility identical with that of the COOH-terminal domain of metallothionein II (α fragment) (Fig. 5). The α domain is known to enfold the 4-metal cluster A (16, 17). Integration of the densitometric scans of the gels showed that the quantity of the α fragment increased in samples binding up to 4 Cd ions per molecule and subsequently decreased concomitantly with an increase in a stained band corresponding to native metallothionein II. These data suggest that binding is ordered and occurs initially in cluster A followed by cluster B. In order to verify that the subtilisin-resistant peptide was the authentic α fragment, the experiment was repeated with isoform I of metallothionein, since the α I fragment exhibits a different electrophoretic mobility than α II. As can be seen in Fig. 6, samples reconstituted with up to 4 Cd ions followed by incubation with subtilisin revealed an increasing quantity of a peptide fragment identical in mobility with that of α I. Binding of Cd in excess of 4 ions per molecule showed a decreasing amount of α I and a concomitant increase in native metallothionein I.

Reconstitution studies of the two isoforms of apometallothionein with Zn ions revealed a similar pattern. Addition of up to 4 eq of Zn\(^{2+}\) followed by proteolysis revealed an increasing concentration of α fragment. Increasing the equivalency above 4 led to an increasing amount of native metallothionein and a corresponding decrease of α fragment.

Bands of intermediate mobility were repeatedly seen in reconstitution experiments with metallothionein I but not metallothionein II. The identity and significance of these bands remain unclear. The bands were apparent only in the electrophoretic separation of proteolyzed or even unproteolyzed reconstitution samples. Resolution of α fragment and intact metallothionein was also achieved by DEAE-cellulose column chromatography. In the chromatography experiments with metallothionein I, bands of intermediate charge were not seen. However, electrophoresis of the renatured native-like metallothionein I isolated by DEAE-cellulose showed bands of lower mobility. The metal binding affinity of metallothionein I is lower than that of metallothionein II, so the presence of additional bands in metallothionein I is probably an artifact of electrophoresis, possibly oxidation or aggregation.

To confirm that the proteolytically resistant peptide observed in samples with 1 to 4 metal ions bound was the α fragment, samples were reconstituted with 4 Cd digested with subtilisin and then chromatographed on Sephadex G-75. The major peptide eluted in the column effluent had a distribution coefficient (K\(_{d}\)) of 0.7, a value identical with that of the Cd\(_{4}\) α domain. The elution profile of Cd was coincident with the peptide absorbance at 230 nm. Cd\(_{4}\)-reconstituted metallothionein eluted with a K\(_{d}\) of 0.54, a value identical with that of native Cd\(_{4}\)Zn metallothionein. Amino acid analysis of the Cd-containing peptide prepared from subtilisin-treated Cd\(_{4}\)-reconstituted apometallothionein I revealed a composition similar to that seen with the α fragment (Table I). The metal content of the peptide averaged 3.8 Cd per molecule.

The peptides generated after digestion of the reconstituted samples with subtilisin were evaluated. Samples reconstituted with 0 to 7 Cd eq and subsequently incubated with the protease were applied to thin layer cellulose for ascending partition chromatography (Fig. 7). The concentration of the peptide mixture visualized with ninhydrin decreased as the number of Cd ions bound increased. Upon saturation of the binding sites in metallothionein, the molecule behaves as native metallothionein in remaining at the origin of chromatography and showing little ninhydrin reactivity. These results corroborate the electrophoretic data and indicate that increasing the number of bound metal ions leads to increasing amounts of polypeptides resistant to proteolysis.

Several experiments were carried out to examine whether α-fragment observed in the above studies resulted from intra-molecular metal ion exchange caused by proteolysis. Apometallothionein II reconstituted with 2 Cd eq and diluted to

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**TABLE I**

| Residue | Cd\(_{4}\)-reconstituted metallothionein I | α-1 fragment (16) |
|---------|-----------------------------------------|-------------------|
| Asp     | 1.4                                     | 1.3               |
| Thr     | 1.1                                     | 1.0               |
| Ser\(^{a}\) | 3.6                                     | 3.8               |
| Glu     | 1.3                                     | 1.4               |
| Pro     | 1.1                                     | 1.1               |
| Gly     | 3.6                                     | 3.5               |
| Ala     | 3.0                                     | 3.2               |
| Cys\(^{b}\) | 11.7                                    | 11.1              |
| Val     | 2.1                                     | 2.1               |
| Lys     | 4.1                                     | 5.2               |

\(^{a}\) Values are from 24-h hydrolysates.

\(^{b}\) Cys determined after performic acid oxidation.
Order of Metal Binding in Metallothionein

13067

FIG. 7. Ascending thin layer chromatography of apometallothionein I reconstituted with 0 to 7 Cd ions per molecule and digested with subtilisin. The protease incubation was in 25 mM NH₄HCO₃, pH 7.8. The samples were lyophilized and resuspended in H₂O 5 times prior to applying 10 nmol of sample to each lane. Both Cd, α and Cd,Zn metallothionein I remained at the origin of chromatography and showed little ninhydrin reactivity.

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concentrations ranging from 0.1 mM to 0.1 μM was incubated with the same concentration of subtilisin for 2 h. Analysis of the products by polyacrylamide gel electrophoresis revealed the presence of α fragment at each dilution. No β fragment (NH₂-terminal domain) was apparent. In a second experiment Cd₇-reconstituted metallothionein II was digested with subtilisin for 10 min in the presence of 5 to 20 mM EDTA. The presence of the chelator had no effect on the appearance of a fragment by electrophoresis and in that time interval had no effect on the absorption properties at 250 nm of Cd₂ metallothionein. EDTA at a final concentration of 5 mM was also incubated with 60 μM Cd₇-reconstituted metallothionein II for 5 to 40 min. At the end of this incubation time, subtilisin was added and proteolysis was allowed to proceed for 10 min. The samples analyzed by electrophoresis showed α fragment and no native metallothionein indicating that EDTA was stripping cluster B ions initially. These experiments suggest that Cd-α is the kinetically and thermodynamically stable intermediate.

Reconstitution experiments were also done with purified α fragment which had been demetalized. Apo-α I was reconstituted with 0 to 4 g atoms of Cd per mol of protein followed by incubation with subtilisin. An aliquot was taken for non-denaturing gel electrophoresis, and recovery was assessed by integration of the densitometric scans of the gels. As can be seen in Fig. 8, a linear relationship was found 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. 8). 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. Proteolytic degradation decreases concomitantly with an increase in the concentration of α. The linearity of these inverse plots and the apparent lack of intermediate peptides are consistent with a cooperative binding of metal ions to cluster A. As such, only saturated α clusters and apoprotein would exist with the concentration of each being dependent on the Cd equivalents added.

If cooperative binding occurs, the initial phase should be a cooperative saturation of cluster A. For example, reconstitution with 2 Cd ions followed by proteolysis should result in 50% of the molecules being recovered as Cd₄ α. Fig. 9A shows the theoretical curves for positive cooperativity in the metal ion binding to the two domains. The two curves represent the recoveries of saturated 4-metal and 3-metal domains. The actual recoveries of the 2 domains based on the starting experiment.
apometallothionein (Fig. 9B) was determined from the amino acid compositions of the reconstituted samples exposed to proteolysis and subsequently gel filtered to separate the combined pool of metallothionein, and α fragment from digested peptides. The domain of metallothionein I-containing cluster A contains all the Ala and Val residues in the metallothionein 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 2 domains are similar to the theoretical values for positive cooperativity in binding. Also shown in Fig. 9B is the fluorescein reactivity curve of the Cd-reconstituted samples. To ensure against only partial digestion, 16 h was allowed for proteolysis. Completion of digestion of apometallothionein, Cd₀ and Cd₄-reconstituted metallothionein occurred in about 2 h of incubation.

Quantitation of the metal content of various proteolyzed reconstituted samples, after separation of metallothionein and α fragment by Sephadex G-75 column chromatography, confirmed that cooperative binding had occurred (Table II). The α fragment recovered from Sephadex G-75 after reconstitution of apometallothionein with only 2 Cd ions per molecule did not show 2 Cd ions bound for each molecule, but rather 4 g atoms of Cd per mol α for half of the molecules. The Cd₀-reconstituted metallothionein was resolved into a major α fraction (3.8 Cd/molecule) and a minor metallothionein peak containing 6.5 Cd per molecule. Likewise, Cd₀-reconstituted metallothionein was resolved into a population of Cd-saturated metallothionein molecules and a population of metal saturated α fragments.

**Table II**

| Cd⁺ added/apo-metallothionein | Sephadex G-75 separated molecules | Metallothionein | α |
|-------------------------------|----------------------------------|---------------|---|
| g atoms Cd/mol                |                                  |               |   |
| 7                             | 6.8                              |               |   |
| 5                             | 6.7                              |               |   |
| 4                             | 6.5                              |               |   |
| 2                             | 4.0                              |               |   |

**DISCUSSION**

Reconstitution studies were undertaken to study the order of filling of the 7 metal sites of metallothionein. Under the *in vitro* conditions employed, the addition of 7 metal ions per molecule of apometallothionein restored native-like properties to the molecule. In addition, concentrations of Cd in excess of 7 ions per molecule did not alter the absorption properties of the molecule. These results suggested that binding was specific. Reconstitution of apometallothionein with less than saturating amounts of Cd or Zn ions showed that metal binding appeared to occur by ordered ligation to cluster A initially and not by random chelation to sites within each domain. These results were based on the proteolytic susceptibility of reconstituted samples. Apothionein is completely digested by subtilisin, whereas protection against proteolysis is afforded by the binding of metal ions. Native Cd⁺,Zn⁺ metallothionein and the isolated Cd₀ α fragment are resistant to proteolysis even after prolonged incubation with the protease. Samples reconstituted with 1 to 4 Cd ions per molecule and then subjected to proteolysis yielded increasing quantities of the α fragment which enforces the 4-metal cluster A. The amino acid composition of this polypeptide was similar to that reported for the α fragment isolated previously (16) except that the lysine content was reduced from 5 to 4 residues per molecule. Since α was shown to encompass the sequence for residues 30 through 61, cleavage between Lys₃₀ and Lys₃₁ would give a peptide whose composition would be compatible with that reported in this study.

Binding of Cd in excess of 4 ions per molecule gave increasing amounts of native-like metallothionein as the 3-metal cluster B became saturated. Thus, metal binding is ordered and an intermediate in the binding process (Cd₂⁺, metallothionein) can be isolated. Cd α is also a stable intermediate when proteolysis of Cd⁺ metallothionein is allowed to proceed in the presence of EDTA. Thus, α fragment is the kinetically and thermodynamically stable intermediate. The binding process in each domain is cooperative. Addition of 1 Cd per molecule of apometallothionein results in approximately 25% of the molecules containing Cd₁ cluster A and 75% of the molecules remaining metal free. Cluster B shows similar cooperativity since addition of 5 Cd ions per molecule results in complete saturation of cluster A and saturation of cluster B in 30% of the molecules. Binding cooperativity is evident within each domain, but no apparent cooperativity exists between domains.

Vasak and Kagi (26) have renatured rabbit liver metallothionein *I in vitro* with Co(II). Their electronic and EPR spectroscopic data not only support the existence of two metal clusters in metallothionein, but also suggest that the mode of formation of the cluster is stepwise. The first 4 metal ions bound produced virtually no magnetic interaction and, therefore, were presumably distributed between the two clusters. This is in marked contrast to the data presented in this report. The remaining 3 Co ions gave increasing interaction as the separate sites became linked yielding Co₁ metallothionein which is diamagnetic presumably due to antiferromagnetic coupling. Vasak and Kagi suggested that the first 4 metal ions produced a steric configuration necessary for binding of the remaining three (26). The formation of the metal thiolate clusters in rat liver metallothionein with Cd or Zn ions is clearly ordered, and the cooperative binding should yield interaction as cluster A is formed. The difference observed between reconstitution with Co and Cd or Zn is perplexing. Experiments with Co(II) are difficult since Co binding to metallothionein requires strict anaerobiosis. We plan to reinvestigate Co binding to apometallothionein and apo-α of rat liver metallothionein.

The 2 domains appear to differ in their metal-binding properties. Metallothionein induced with Cd is isolated with the Zn ions being preferentially in cluster B (14, 15). This distribution is presumably a result of the higher binding affinity apparent in cluster A and the higher association constants known for Cd compared to Zn with thiolate ligands. Metallothionein isolated from calf liver contains Cu(I) preferentially in cluster B with Zn ions ligated in cluster A (20). The order of binding of Cu to metallothionein will have to be studied to determine if the order of cluster filling differs from that seen with Cd or Zn. Metallothionein induced by Cu in rat liver was shown to bind 9 to 11 g atoms of Cu per mol of protein with the Cu ions being diamagnetic (21). The structure of the clusters may be altered to accommodate the increased number of metal ions bound. The possibility exists that differences in the cluster structure or the distribution of Zn and Cu in different domains may alter the function of metallothionein in Cu or Zn metabolism or homeostasis.

The release of metal ions for metallothionein may also be cooperative. If the release corresponds to binding affinity, then the order of release would be cluster B metal ions prior to those in cluster A. The release of 3 or 4 metal ions may be
an important feature in the still unresolved function of metallothionein.

REFERENCES

1. Kagi, J. H. R., Kojima, Y., Kissling, M. M., and Lerch, K. (1980) Ciba Found. Symp. 72, 223–237
2. Nordberg, M., and Kojima, Y. (1978) in Metallothionein (Kagi, J. H. R., and Nordberg, M., eds) pp. 41–117, Birkhäuser Verlag, Basel, Switzerland
3. Kagi, J. H. R., and Vallee, B. L. (1960) J. Biol. Chem. 235, 3460–3465
4. Pulido, P., Kagi, J. H. R., and Vallee, B. L. (1966) Biochemistry 5, 1768–1777
5. Tsunoo, H., Kino, K., Nakajima, H., Hata, A., Huang, L.-Y., and Yoshida, A. (1978) J. Biol. Chem. 253, 4172–4174
6. Buhler, R. H. O., and Kagi, J. H. R. (1974) FEBS Lett. 39, 229–234
7. Winge, D. R., Premakumar, R., and Rajagopalan, K. V. (1975) Arch. Biochem. Biophys. 170, 242–252
8. Weser, U., Rupp, H., Donay, F., Linnemann, F., Voelter, W., Voetsch, W., and Jung, G. (1973) Eur. J. Biochem. 39, 127–140
9. Nordberg, G. F., Norberg, M., Piscator, M., and Vesterberg, O. (1972) Biochem. J. 126, 491–498
10. Karin, M., and Herschman, H. R. (1980) Eur. J. Biochem. 107, 395–401
11. Durnam, D. M., Perrin, F., Gannon, F., and Palmiter, R. D. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 6511–6515
12. Enger, M. D., Rall, L. B., and Hildebrand, C. E. (1979) Nucleic Acids Res. 7, 271–288
13. Huang, L.-Y., Kimura, M., Hata, A., Tsunoo, H., and Yoshida, A. (1981) J. Biochem. (Tokyo) 89, 1839–1845
14. Otvos, J. D., and Armitage, I. M. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 7094–7098
15. Otvos, J. D., and Armitage, I. M. (1981) in Biochemical Structure Determination by NMR (Sykes, B. D., Glickson, J., and Borther-By, A. A., eds) Marcel Dekker, New York
16. Winge, D. R., and Miklosy, K.-A. (1982) J. Biol. Chem. 257, 3471–3476
17. Boulanger, Y., Armitage, I. M., Miklosy, K.-A., and Winge, D. R. (1982) J. Biol. Chem. 257, 13717–13719
18. Melis, K. A., Carter, D. C., Stout, C. D., and Winge, D. R. (1983) J. Biol. Chem. 258, 6255–6257
19. Boulanger, Y., Goodman, C. M., Forte, C. P., Fesik, S. W., and Armitage, I. M. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 1501–1505
20. Briggs, R. W., and Armitage, I. M. (1982) J. Biol. Chem. 257, 1259–1262
21. Winge, D. R., Geller, B. L., and Garvey, J. (1981) Arch. Biochem. Biophys. 208, 160–166
22. De Bernardo, S., Weigele, M., Toome, V., Manhart, K., Leimgruber, W., Bohlen, P., Stein, S., and Udenfriend, S. (1974) Arch. Biochem Biophys. 163, 390–399
23. Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121, 404–427
24. Kagi, J. H. R., and Vallee, B. L. (1961) J. Biol. Chem. 236, 2435–2442
25. Vasak, M., Kagi, J. H. R., and Hill, H. A. O. (1981) Biochemistry 20, 2852–2856
26. Vasak, M., and Kagi, J. H. R. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 6709–6713
Order of metal binding in metallothionein.
K B Nielson and D R Winge

J. Biol. Chem. 1983, 258:13063-13069.

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