Stoichiometry and cluster specificity of copper binding to metallothionein: homogeneous metal clusters

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Experiments were done to define the stoichiometry of binding of Cu(I) to metallothionein (MT) and to determine its sites of binding in mixed-metal species. Spectrophotometric titrations of rabbit liver Cd_7-MT 2, apoMT, and Cd_4-α-domain with Cu(I) revealed endpoints of 3–4, 4 and 8, and 4 and 6–7 added Cu(I)/mol of MT for the three species respectively. Observed endpoints depended on conditions of the titration and the wavelength chosen for absorbance measurement. Nevertheless, from metal and sulphydryl analyses of titrated proteins that were pretreated with Chelex-100 to remove metal ions from solution, almost all of the cadmium was displaced from Cd_7-MT by the addition of 7 Cu(I)/mol of MT. Similarly, 4 Cu(I)/mol of Cd_4-α-domain completely displaced bound cadmium. The Cu_4-α-domain was converted into a Cu_6-α species upon addition of two equivalents of Cu(I)/mol of α-domain. Reaction of Cd_7-MT with 7, 12 and 20 Cu/mol of MT, followed by reaction with Chelex resin, generated protein samples in each case with about 7 Cu/mol of MT. ^{111}Cd-NMR analysis of the reaction of $^{111}Cd_7$-MT with Cu(I) showed that nearly co-operative one-for-one replacement of ^{111}Cd occurred and that the β-domain cluster reacted before the α-domain cluster. Two mixed-metal MTs with Cu to Zn ratios approximating 3 to 4 and 6 to 4 were isolated from calf liver. After substitution of Zn with ^{111}Cd, NMR spectra of each protein showed that ^{111}Cd was confined almost completely to the α-domain. By inference, about 3 or 6 Cu were bound in the β-domain of these proteins. Supporting this segregation of metal ions into domains, reaction of Cu_6,Zn_4-MT with nitrilotriacetate removed zinc exclusively, whereas reaction of Cu_6,Cd_4-MT with 4,7-phenylsulphonyl-2,9-dimethyl-1,10-phenanthroline extracted only Cu(I). Proteolytic digestion of both products followed by gel filtration demonstrated that Cu(I) and Cd were bound to fragments of the intact protein. Finally, reaction of rabbit liver $^{111}Cd_7$-MT 2 with Cu_{10}-MT 2 resulted in interprotein metal exchange in which ^{111}Cd moved from the β- to the α-domain according to NMR analysis. In contrast with the prevalent view that six Cu(I) bind to each domain of MT, the present results show that Cu(I) binds to MT with a minimum stoichiometry of about 7 Cu(I)/mol of MT and can bind to the α-domain with stoichiometries of 4 or 6 Cu(I)/mol of MT. Although MTs interacting with 12 or 20 Cu(I)/mol of MT are less stable than that binding about 7 Cu(I)/mol, it appears that MT can bind Cu(I) in multiple stoichiometries.

INTRODUCTION

A number of studies of the stoichiometry of binding of copper to mammalian metallothionein (MT) have produced variable results, ranging from 7 to 15 or more Cu per mol of protein [1]. In some cases titration experiments were carried out, in which Cu-containing MTs were generated *in vitro* [2–6]. Generally, endpoints were defined by breaks in spectrophotometric or CD titration plots, not by measurement of metals bound to the protein. An investigation by the present authors into the metal-to-protein stoichiometry of native Cu,Zn-MT samples from calf liver indicated that MT may adopt a range of several metal-to-protein stoichiometries [1]. Thus, the variability among *in vitro* results may not be artifactual but, instead, may reflect the diversity of forms of Cu-containing MT that do exist *in vivo*.

Studies of the *in vitro* formation of Cu-MT have largely used apo- or Zn-MT as the starting form of the protein [2–6]. In some cases, subtilisin was present to digest residual apoprotein or other reactive forms of MT unsaturated with Cu protein [2–4]. In these latter studies, Cu appeared to react co-operatively first with the β-domain of MT to form a Cu_6S_9 metal–thiolate structure which was stable in the presence of subtilisin. Once this part of the molecule had been titrated, Cu entered the α-domain co-operatively to constitute a Cu_6S_{11} cluster.

Because of the finding of widely varying Cu to Zn ratios in naturally occurring calf liver MTs, studies were undertaken to examine how these metal ions were distributed among the two metal-binding domains of mammalian MTs and how these species might be generated. Analysis of metal distribution among clusters in the present experiments depended upon the fact that Cd can replace Zn but not Cu(I) one-for-one in MT, that Cu^+ stoichiometrically replaces Cd in the protein, and that ^{111}Cd-NMR studies provide direct information about the location of $^{111}Cd^{2+}$ ions in the two clusters [7,8].

MATERIALS AND METHODS

Reagents

CdO, enriched in ^{111}Cd, was purchased from Oak Ridge National Laboratories. Tetrakis(acetonitrile) copper(I) hexafluorophosphate {$[Cu(NCCH_3)]_4PF_6$} was obtained from Aldrich Chemical Company. Subtilisin (peptidase type VIII) was a product of the Sigma Chemical Company. Other common chemicals were reagent grade or the highest purity available.

Isolation of MTs

Cu-containing MTs were purified by the same methods as described in the preceding paper [1]. Zn-MTs were isolated by similar procedures under aerobic conditions from the livers of male New Zealand White rabbits or male Harlan Sprague–

Abbreviations used: BCS, 4,7-phenylsulphonyl-2,9-dimethyl-1,10-phenanthroline; NTA, nitrilotriacetate; $[Cu(NCCH_3)]_4PF_6$, tetrakis(acetonitrile) copper (I) hexafluorophosphate; MT, metallothionein (numbers of bound metals are specified by numerical subscripts, whereas the cluster is designated by a subscript α or β).

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Dawley white rats, which were injected with 1 mg/kg per day of $ZnSO_4$ for 7 days. About 100 g of liver was homogenized in 200 ml of 5 mM Tris/HCl buffer with 6 mM 2-mercaptoethanol, pH 8.0, and centrifuged at 48000 ***g*** for 60 min. The supernatant was loaded on to a 12 cm × 85 cm Sephadex G-75 column and eluted with 5 mM Tris/HCl, 2 mM 2-mercaptoethanol, pH 8.0. The MT fractions, located by atomic absorption spectrophotometry, were applied to a DEAE A-25 ion-exchange column and eluted with a 2 litre gradient of 5 to 300 mM Tris/HCl, pH 7.8, at 4 °C.

Isolation of the Cd_4-α-domain

A method described by Savas et al. was followed to isolate Cd_4-α [9]. Zn_7-MT was reacted with 4.5 mol equiv. of Cd(II) and a slight excess of EDTA under an Ar atmosphere at room temperature [9]. After 1.5 h of incubation, subtilisin was added to the solution and incubated for 20 h under the same conditions. In order to separate the Cd_4-α-domain from the other products of the reaction, the resultant solution was loaded into a Sephadex G-25 column (1.5 cm × 177 cm) using 5 mM Tris buffer as eluant buffer. To locate the Cd_4-α-domain, the fractions were analysed for cadmium content using atomic absorption spectrophotometry.

Formation of $^{111}Cd_7$-MT from rabbit and calf liver MTs

$^{111}CdCl_2$ was made by dissolving ^{111}CdO in conc. HCl. After much of the HCl had evaporated on a steam bath and the pH of this resultant solution was raised to 4.4, ^{111}Cd-MT was made by replacing Zn in the protein with ^{111}Cd. The $^{111}CdCl_2$ solution was added dropwise with stirring to a well-buffered solution of about 0.1 mM MT. Chelex-100 resin was added to remove free Zn and excess ^{111}Cd.

Production of apoMT

The pH of a solution of 7.4 μM Zn_7-MT 2 was reduced to 3.0 to dissociate Zn^{2+} from Zn_7-MT. In order to separate the apoMT from other products of the reaction, this acidic solution was loaded on to a Sephadex G-25 column using 0.05 mM HCl as eluant buffer. Fresh apoMT was used in titration studies after its pH was raised to pH 7.4 with solid Tris.

Synthesis of $[Cu(I)(NCCH_3)]_4ClO_4$

$[Cu(NCCH_3)]_4PF_6$ used in the spectrophotometric titrations was purchased from Aldrich Chemical Co. That employed in the NMR titration of Cd_7-MT was prepared by the method of Hemmerich and Sigwart [10]. The crystals obtained were dissolved anaerobically in CH_3CN/water (1:1, v/v). The resultant solution was stored in an inert atmosphere to prevent oxidation. Its concentration of Cu was determined by atomic absorption spectrophotometry. Less than 1 part per 40000 parts of total Cu was detected as Cu^{2+} by ESR spectroscopy. In contrast, anaerobic solutions of CuCl contained much more Cu^{2+} according to ESR spectral analysis.

Spectrophotometric titration of MTs with $Cu(I)(NCCH_3)_4^+$

In an anaerobic chamber 2 μl aliquots of $[Cu(NCCH_3)]_4PF_6$ prepared in 2% acetonitrile solution were added to either Cd_7-MT, Cd_4-α-domain or apoMT. After each addition, the anaerobic cuvette was sealed and the spectrum of its contents recorded over the wavelength range of 190–400 nm, in a Perkin–Elmer Lambda 6 spectrophotometer at 25 °C. All of the samples were analysed against blanks containing the same concentration of all reactants excluding the protein. Each titration was carried out three times with closely similar results.

In some cases, Chelex-100 metal-chelating resin was added to samples to remove loosely bound or displaced metal ions. The remaining solutions were assayed for metal ions by atomic absorption spectrophotometry and for sulphydryl groups with 5,5′-dithiobis-(2-nitrobenzoate) [1]. The sulphydryl concentration divided by 20 was defined as the molarity of MT protein. The UV spectra of some of these solutions were also measured.

^{111}Cd-NMR experiments

^{111}Cd-NMR spectra were recorded at 106 MHz on a General Electric GN-500 spectrometer. The broadband ^{1}H-decoupler was turned on during data acquisition and turned off during the delay period to obtain decoupled spectra without nuclear Overhauser effects. MT samples were contained in 10-mm-bore tubes with 10–15% ^2H_2O for the field-frequency lock. Chemical shifts are reported in p.p.m. downfield from the ^{111}Cd resonance of 0.1 M $Cd(ClO_4)_2$.

Proteolytic digestion of MT

Cu_6,Cd_4-MT (90 μM) was reacted anaerobically with 40 mM 4,7-phenylsulphonyl-2,9-dimethyl-1,10-phenanthroline (BCS) overnight to remove Cu. Then 4.5 μM subtilisin was added. Digestion of the protein was carried out anaerobically for at least 17 h. The product was applied to a Sephadex G-50 gel-filtration column (1.0 cm × 120 cm) and eluted with 10 mM NH_4HCO_3 at pH 7.8. The fractions were collected and measured by atomic absorption spectrophotometry. Similarly, Cu_6,Zn_4-MT was incubated with 30 mM nitrilotriacetate (NTA) to extract Zn, then treated with subtilisin, and chromatographed as above.

RESULTS

Spectrophotometric titration of MTs with $Cu(I)(NCCH_3)_4^+$

Spectrophotometric titrations were carried out to assess the validity of this type of experiment as a tool to define the stoichiometry of binding of Cu^+ to MT. In contrast to earlier studies, after the titration Chelex resin was added to remove metal ions not firmly bound to the protein. Then, metal to protein stoichiometries were measured by atomic absorption spectrophotometry and thiol determinations.

Cd_7-MT 2 from rabbit liver was titrated under anaerobic conditions with $Cu(I)(NCCH_3)_4^+$. To follow the progress of the reaction the UV spectrum of the reaction mixture was recorded after each addition of Cu(I) (Figure 1, upper panel). As Cu(I) displaced Cd from the protein structure, absorbance above 250 nm increased as Cu(I)–thiolate bands formed. An isosbestic point at 250 nm was observed between 0 and 3–4 Cu per 7 Cd. According to the summary of this experiment in Figure 1 (lower panel), when absorbance changes were monitored at 250 or 280 nm as a function of added Cu(I), the only break occurred at 3–4 added Cu(I) per mol of MT. The progressive increase in absorbance from 4 to 14 Cu added per mol of MT shown in Figure 1 (upper panel) indicated that Cu(I) continued to form interactions with MT well beyond the one endpoint that was detected.

The extent of replacement of Cd by Cu(I) in the protein was assayed after treating samples with Chelex-100 metal-chelating resin to remove unbound or loosely bound metal ions. The resin was allowed to settle out after 30 min incubation with stirring, and the metals remaining in solution were measured by atomic absorption spectrophotometry. Upon the addition of 4 or 7 Cu(I)

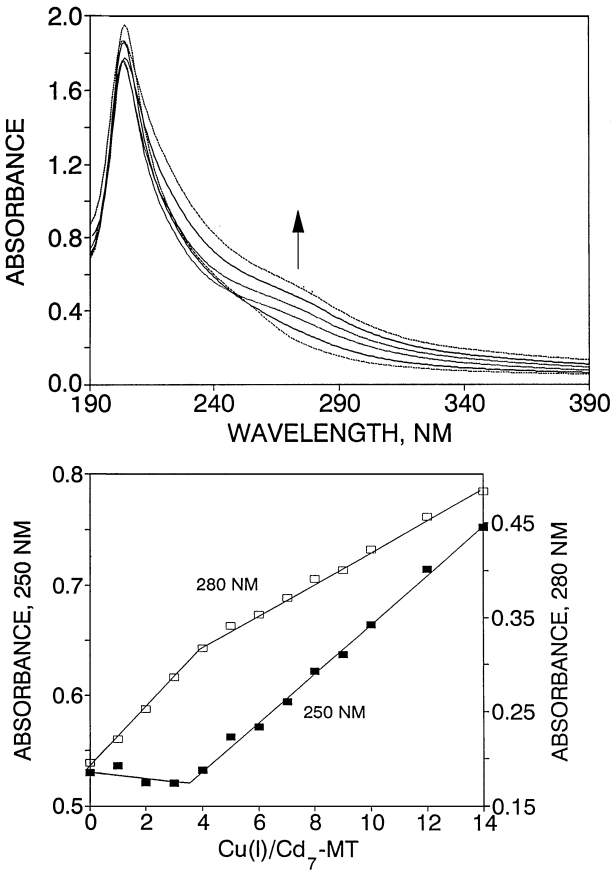

Figure 1 Spectrophotometric titration of rabbit liver Cd_7-MT 2 with $Cu(I)(NCCH_3)_4^+$

Reaction of 13.2 μM Cd_7-MT 2 was carried out anaerobically at 25 °C and pH 7.4 in 10 mM phosphate buffer. Upper panel: titration spectra. The arrow indicates the direction of the change in absorbance with added Cu. The lines indicate the addition of 0, 2, 4, 7, 10 or 14 molar equivalents of Cu to the Cd_7-MT. Lower panel: changes in absorbance at 250 and 280 nm as a function of added Cu(I).

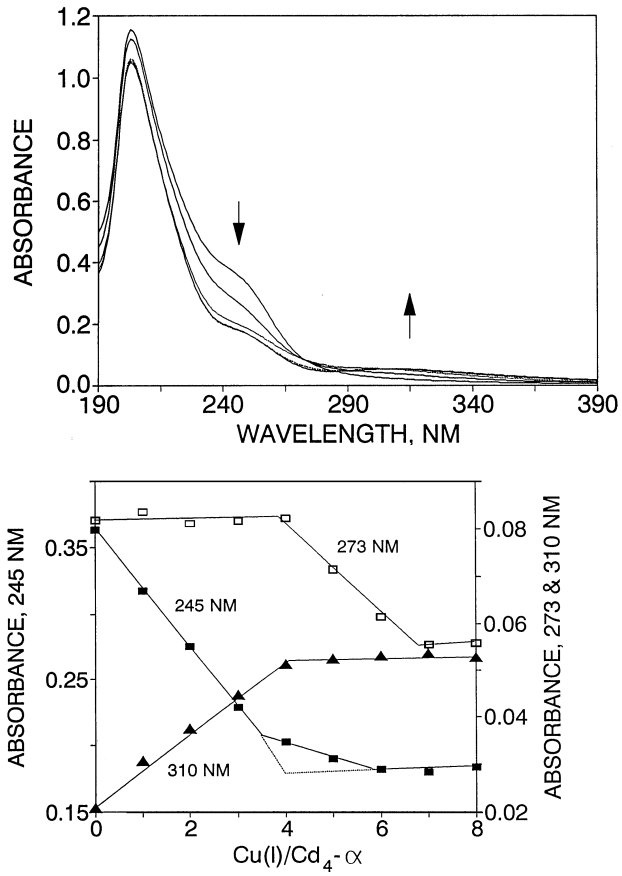

Figure 2 Spectrophotometric titration of rabbit liver Cd_4-α-domain with $Cu(I)(NCCH_3)_4^+$

Reaction of 8.4 μM Cd_4-α-domain was carried out anaerobically at 25 °C and pH 7.4 in 10 mM phosphate buffer. Upper panel: titration spectra. The arrows indicate the direction of the change in absorbance with added Cu. The lines indicate the addition of 0, 2, 4, 6 or 8 molar equivalents of Cu to the Cd_4-MT; the lines indicating the addition of 6 or 8 molar equivalents are superimposed. Lower panel: changes in absorbance at 245, 273 and 310 nm as a function of added Cu(I).

per mol of MT, 3.8 and 6.2 g-atoms of Cd/mol were lost and 3.9 and 7.1 g-atoms of Cu/mol were added to the protein, respectively. In results not shown, 7 g-atoms of Cu/mol of MT fully displaced Zn from Zn_7-MT, according to measurements of Zn released from the protein. Thus, the spectrophotometric titration did not reveal the point of complete displacement of Cd by Cu determined by direct measurement of protein-bound metal.

When Cd_4-α was titrated with Cu(I), absorbance bands at 250 and 310 nm were generated (Figure 2, upper panel). An isosbestic point at 273 nm characterized spectra of solutions titrated with the addition of 0 to 4 Cu/mol of MT. It was also evident that most of the changes in absorbances occurred in this part of the titration. According to Figure 2 (lower panel), endpoints of 3–4 and 6–7 added Cu/mol of MT were observed at different wavelengths. In addition, after removal of unbound metal ions with Chelex resin, it was shown by atomic absorption spectrophotometry that 4 Cu(I) ions had displaced all 4 Cd^{2+} ions and upon total addition of 6 Cu^+, 5.8 Cu/mol of MT were bound. These results are consistent with the successive occupation of the α-domain of MT with a total of 4, and 6 Cu(I) ions.

In Figure 3 (upper panel) the spectral changes occurring during the titration of apoMT 2 with the Cu(I) reagent are summarized. With $Cu(NCCH_3)_4^+$ added to both sample and reference there is a broad increase in absorbance centring on 270 nm and extending out well above 300 nm. As summarized in Figure 3 (lower panel), the titration observed at 265 and 300 nm revealed breakpoints at 4 and 8 Cu/mol of MT. Interestingly, different endpoints were revealed by absorbance measurements at 265 nm and 300 nm. Thus, the choice of wavelength for analysis is directly related to the conclusions that are derived from the experiment. As in Figure 1 (upper panel), the continual increase in absorbance across the spectrum up to the addition of 16 Cu/mol of MT signalled that Cu(I) bound to the protein beyond the observed break points in the titration.

It is evident that experimental conditions and the choice of wavelength for observation of the reactions alter the detailed outcomes of these spectrophotometric titrations. Nevertheless, in general, endpoints have been observed at about 3–4 and 7–8 Cu/mol of MT. Little indication of an endpoint at 12 Cu/mol was obtained.

Because of the observation that absorbance increases occurred that were not capped by titration endpoints, an experiment was designed to measure the stoichiometry of firmly bound Cu to MT. Cd_7-MT 2 was also reacted with 7, 12, and 20 equivalents of Cu/mol of MT at pH 7.2 for 30 min. The spectra of the

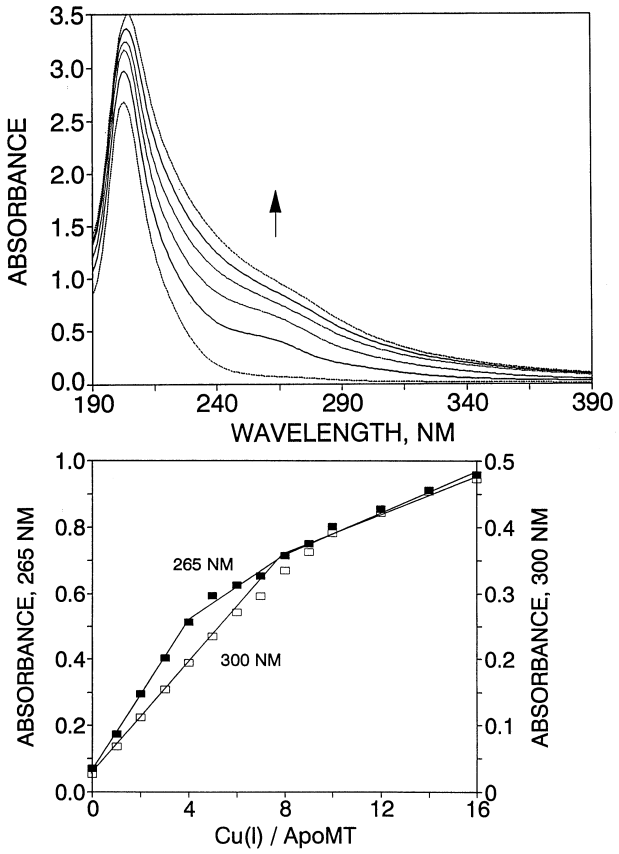

Figure 3 Spectrophotometric titration of rabbit liver apoMT 2 with $Cu(I)(NCCH_3)_4^+$

Reaction of 13.8 μM apoMT 2 was carried out anaerobically at 25 °C and pH 7.4 in 10 mM phosphate buffer. Upper panel: titration spectra. The arrow indicates the direction of the change in absorbance with added Cu. The lines indicate the addition of 0, 3, 6, 9, 12 or 16 molar equivalents of Cu to the apoMT. Lower panel: changes in absorbance at 265 nm and 300 nm as a function of added Cu(I).

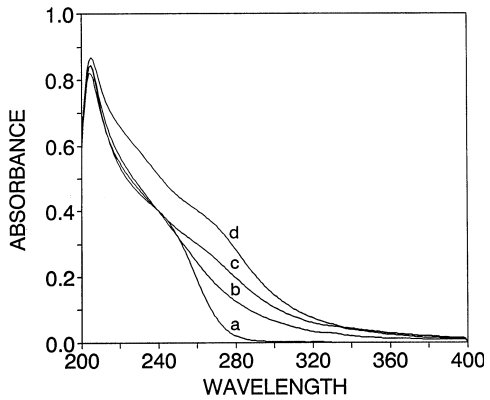

Figure 4 Spectrophotometric titration of rabbit liver Cd_7-MT with $Cu(1)(NCCH_3)_4^+$ in 25% CH_3CN

Cd_7-MT (2.86 μM) (a) was reacted with 7 (b), 12 (c) and 20 (d) equiv. of Cu(I) anaerobically at 25 °C and pH 7.4 in 5 mM Tris/HCl.

product mixtures of these reactions, shown in Figure 4, indicate that the reaction of Cu(I) with the protein occurred up to at least 20 added Cu(I)/mol of MT. Then each sample was stirred with Chelex resin for 5 min, the resin removed by centrifugation, and the supernatant analysed for metals. In each case, all Cd^{2+} was removed, leaving 6.9, 7.2 and 7.2 g-atoms of Cu/mol of MT, respectively, in the MT samples reacted with 7, 12 or 20 Cu/mol of MT.

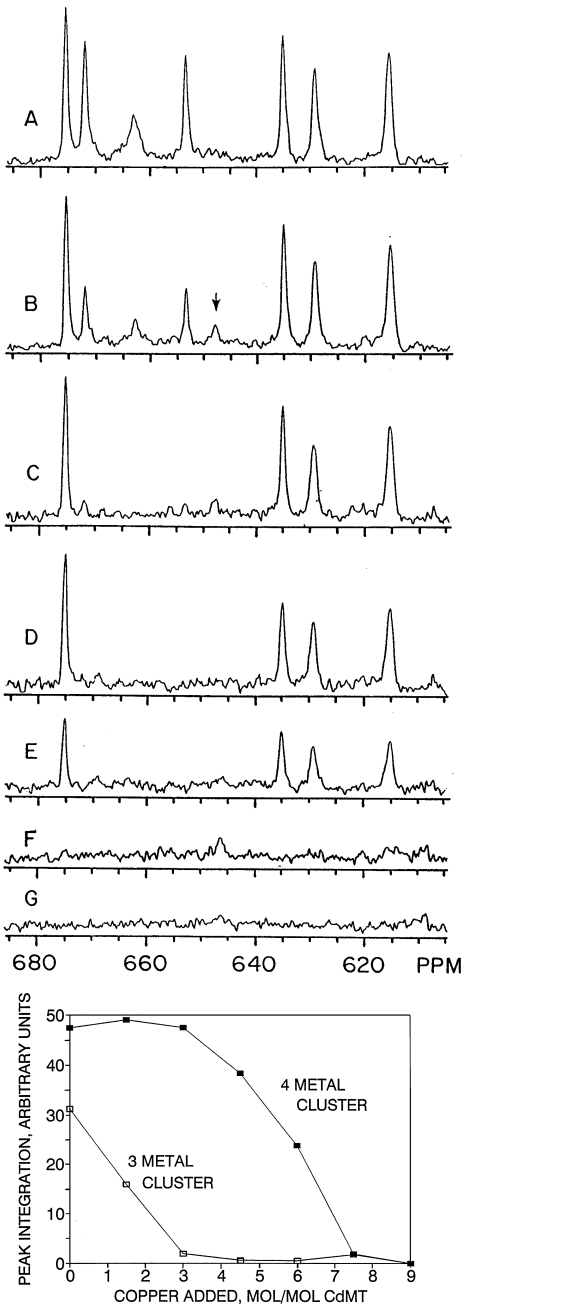

Figure 5 NMR spectra (upper panel) of the titration of rabbit liver $^{111}Cd_7$-MT 2 with $Cu(I)(NCCH_3)_4^+$ and summary of the peak changes (lower panel) for the two clusters, 3-metal cluster (□) and 4-metal cluster (■)

Upper panel: A–G are spectra taken after sequential additions of 1.5 equivalents of Cu(I)/mol of MT. Arrow refers to new ^{111}Cd peak.

NMR titration of $^{111}Cd_7$-MT 2 with $Cu(I)(NCCH_3)_4^+$

To explore how Cu(I) distributes among the clusters of MT, rabbit liver $^{111}Cd_7$-MT 2 was titrated with $Cu(I)(NCCH_3)_4^+ClO_4^-$. Figure 5 (upper panel) shows the ^{111}Cd-NMR spectra of such a

Table 1 Metal to protein stoichiometries in Cu,Zn- and Cd-substituted Cu,Cd-MTs

	[Cu] (mM)	[Zn] (mM)	[Cd] (mM)	Cu/Zn	Cu/(Cd + Zn)
MT 1*	0.48	0.37		1.29	
MT 1†	0.44	0.029	0.26		1.49
MT 1‡	1.92	0.033	1.24		1.52
MT 2§				1.46	
MT 2‖					0.71

* Source for NMR sample.
† Sample *a* upon addition of slight excess of Cd^{2+} relative to Zn^{2+} followed by Chelex treatment.
‡ NMR sample after concentration.
§ From the same liver as MT 1.
‖ MT 2 from a second liver without detectable Zn after Cd substitution.

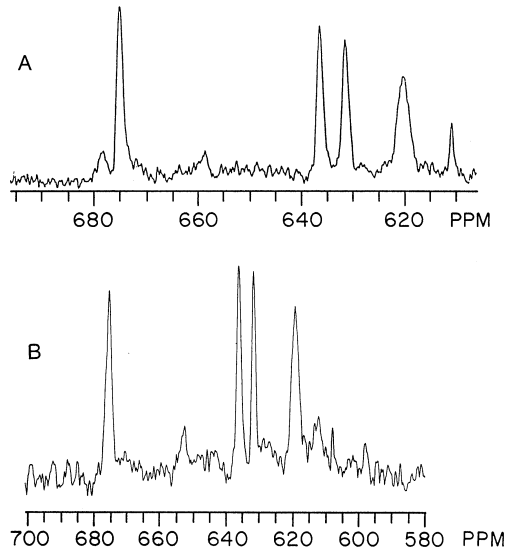

Figure 6 NMR spectra of calf liver Cu,^{111}Cd-MT

(**A**) Cu_3,$^{111}Cd_4$-MT 2, 8.4 mM at 20 °C and (**B**) Cu_6,$^{111}Cd_4$-MT 1, 1.3 mM at 25 °C. Samples were kept in 20 mM Tris/HCl buffer at pH 7.4.

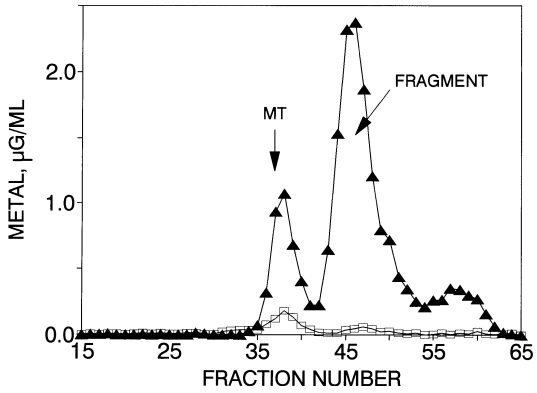

Figure 7 Sephadex G-50 chromatographic separation of products of the sequential reaction of calf liver Cu_6,Cd_4-MT 1, with BCS and then subtilisin

(▲), Cd; (□) Cu. MT (90 μM) was reacted with 40 mM BCS. Cu(BCS) binds to Sephadex and elutes beyond tube 61.

titration. It is evident that at first the Cu(I) reacted exclusively with the 3-metal cluster of $^{111}Cd_7$-MT. The three resonances associated with Cd ions bound in the β-domain of the protein were lost in tandem as Cu(I) was added to $^{111}Cd_7$-MT 2. It is noted that during this part of the reaction, a new peak at 648 p.p.m. appeared, having integrated intensity of about 5% of the original ^{111}Cd intensity in the β-domain, and then disappeared with the addition of more Cu(I). These changes were followed by the co-operative abolition of the resonances from the 4-metal cluster. With the minor exception described above, no perturbations in peak position of ^{111}Cd in the two domains were observed during the titration consistent with co-operative, all-or-nothing, exchange of Cd and Cu in the titration and with the structural independence of the two domains. When the data were summarized in the titration plot of Figure 5 (lower panel), it was evident that Cu(I) had completely titrated the β-domain Cd-cluster at 3 Cu(I) per g-atom of ^{111}Cd. However, the titration of the α-domain resonances occurred non-linearly between 3 and 7.5 Cu(I) per 7 g-atoms of ^{111}Cd. These results suggest that the β-domain initially exchanges Cu for Cd one-for-one, but then may bind a fourth Cu ion before one-for-one exchange of Cu for Cd occurs in the α-domain.

Distribution of Cu(I) in Cu_3,Zn_4-MT 2 and Cu_6,Zn_4-MT 1

From an array of Cu,Zn-MTs purified from calf liver, two were characterized which had Cu to Zn ratios suggestive of segregation of the metals between the two metal clusters, principally as Cu_3,Zn_4-MT 2 and Cu_6,Zn_4-MT 1 species. ^{111}Cd was added to each protein sample to displace bound zinc. According to Table 1, the displacement reaction occurred with stoichiometry closely approximating one-to-one, leaving the copper content of the proteins intact. Total sulphydryl to metal ratios also remained essentially unchanged.

The ^{111}Cd-NMR spectrum of each protein is shown in Figure 6. Both samples displayed a set of resonances characteristic of an intact Cd_4S_{11} α-domain cluster as previously described [11]. Each was missing peaks derived from the Cd_3S_9 clusters in the β-domain. In addition, there were minor resonances in these spectra that do not correspond to peaks in the $^{111}Cd_7$-protein. These are assumed to derive from mixed Cu,^{111}Cd cluster species. Nevertheless, the preponderant location of Cd was in homogenous $^{111}Cd_4S_{11}$ units. By inference, in these protein samples Cu occupied the β-domain and existed in clusters with primarily two stoichiometries, Cu_3S_9 and Cu_6S_9.

Further support for the segregation of the metals in Cu_6,Cd_4-MT 1 among the two domains came from an experiment in which BCS was first reacted with MT to remove bound Cu(I) and then subtilisin was added to digest the metal-depleted parts of the structure. When the products were chromatographed over Sephadex G-50, the profile of metals seen in Figure 7 was

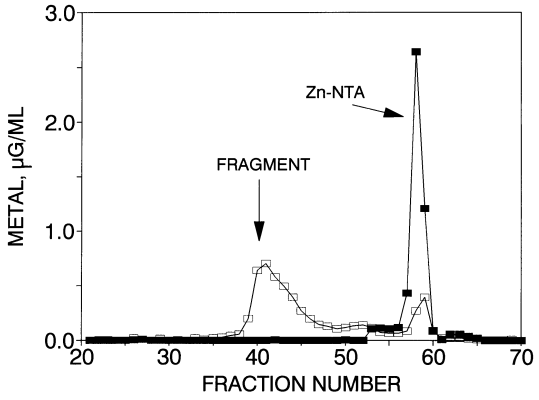

Figure 8 Sephadex G-50 chromatographic separation of products of the sequential reaction of Cu_6Zn_4-MT 2 from calf liver with NTA and then subtilisin

MT (90 μM) was reacted with 30 mM NTA. (■) Zn, (□) Cu.

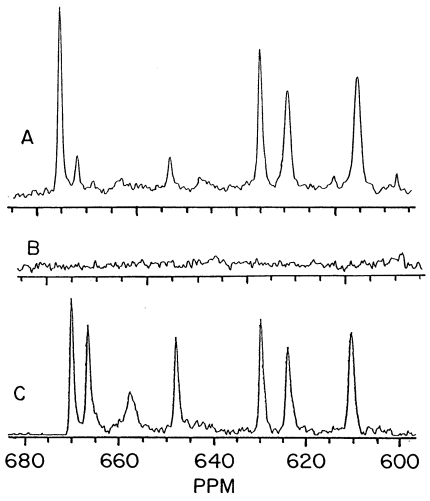

Figure 9 Reaction of rabbit liver proteins $^{111}Cd_7$-MT 2 and Cu_{10}-MT 2 followed by ^{111}Cd-NMR spectroscopy

^{111}Cd was 5.3 mM and Cu was 4.5 mM in 20 mM Tris/HCl at pH 7.4 and 35 °C. (**B**) Cu_{10}-MT 2, (**C**) $^{111}Cd_7$-MT 2 and (**A**) the mixture after reaction of (**B**) and (**C**) for 3 h.

observed in several experiments. There was a small band of Cd and Cu, which migrated as the native protein, and a second, major band containing only Cd. This slower moving fragment appeared to be a MT domain, but its firm identification has not yet been made. Together with the NMR results shown in Figure 6(B), this finding is consistent with the localization of Cd principally in the α-domain and Cu in the β-domain. Interestingly, a small amount of Cu did not react with BCS and could be isolated with holoprotein. Possibly, it was located in mixed Cu,Cd clusters which were resistant to reaction with BCS, as hypothesized for the native Cu,Zn-protein [1].

Reaction of Cu_6Zn_4-MT 1 with NTA and then subtilisin followed by Sephadex G-50 chromatography also led to the elution of a fragment of the protein followed by Zn bound to NTA. In this case it contained only Cu (Figure 8). This finding also supports the view that Cu and Zn were segregated in separate clusters in the holoprotein.

Interprotein metal exchange to form Cu,Cd-MT

To probe other possible mechanisms of formation of Cu,Zn-MT species, rabbit liver $^{111}Cd_7$-MT 2 was mixed with Cu_{10}-MT 2, made by the displacement of Cd from the Cd_7 protein by $Cu(I)(NCCH_3)_4ClO_4$. It was hypothesized that as with mixtures of Zn_7- and Cd_7-MT metal exchange would occur between proteins to generate heterogeneous Cu,Cd-MT [8]. Figure 9 summarizes the experiment. It is evident that peak areas of resonances 1, 5, 6, and 7 increased (1.4 times, by integration) and those of 2, 3, and 4 declined (2 and 4 decreased by 0.7 by integration) after Cu_{10}-MT reacted with Cd_7-MT for a period of 3 h, including spectral acquisition time of 2 h. The peak integrations verify that Cd left the β-domain and appeared in the α-domain cluster. Thus, Cu(I) shifted from α- to β-domains while ^{111}Cd underwent exchange in the opposite direction. Further experiments are underway to fully characterize this interchange process.

DISCUSSION

A survey of the metal-to-protein stoichiometry of a number of Cu,Zn-MTs isolated from bovine calf liver showed that the total metal content of the proteins varied from 7 to about 15 [1]. Those with smaller numbers of metals contained predominantly zinc; those with larger amounts of metal bound more copper. The present experiments were designed to examine the disposition of metals in copper-containing MTs in more detail.

The titration of rabbit liver Cd_7-MT 2 with $Cu(I)(NCCH_3)_4^+$, displayed in Figure 1, indicated an endpoint at 3–4 Cu/mol of MT, with almost all of the Cd displaced at a ratio of 7 Cu/mol of MT according to metal analysis that was not noticeable in the spectral titration. When titration conditions were altered to include a large concentration of CH_3CN as used previously, a number of endpoints were observed depending on the wavelength, including 4–5, 8–9, and 9–10 (results not shown) [5]. Considering the strong preference of Cu(I) for the β-domain as shown in the NMR titration of Figure 5, the spectrophotometric results are consistent with the formation of a succession of species starting with $(Cd_4)_\alpha(Cd_3)_\beta$, followed plausibly by $(Cd_4)_\alpha(Cu_3)_\beta$; $(Cd_4)_\alpha(Cu_{3-4})_\beta$; $(Cu_4)_\alpha(Cu_{3-4})_\beta$; $(Cu_6)_\alpha(Cu_{3-4})_\beta$ or $(Cu_4)_\alpha(Cu_6)_\beta$ and, finally, $(Cu_6)_\alpha(Cu_6)_\beta$, although the last species has not been detected by this method. Nevertheless, it is evident from the experiments that the choice of wavelength to monitor the reaction as well as the conditions of the titration contribute to the observed results. As such, spectral titrations alone appear to be unreliable as a sole means to follow the binding of Cu(I) to MT.

Metal analyses of MT products after titration with Cu(I) established that there is one-for-one displacement of Cd by Cu when 7 equivalents of Cu/mol of MT were added to the protein (Figures 1 and 3). When more Cu(I) was added as in Figure 4, only Cu_7-MT was observed after addition of Chelex to remove free or loosely bound Cu(I). Because of the increasing absorbance of the reaction mixture with each addition of Cu(I), it was plausible that MT species containing more than 7 Cu ions had formed. However, these appear to be readily converted into Cu_7-MT by Chelex. Another recent article examined the reaction of Zn_7-MT with Cu(I) and concluded that Cu(I) forms a continuous range of mixed-metal species, Zn_n,Cu_{12-m}-MT in which n decreases from 7 to 0 as m increases from 0 to 12 [12]. This type of behaviour was not observed with Cd_7-MT as described in the present study nor in unpublished results of Zn_7-MT titrations. Why there is a dramatic difference in results between the two studies is unclear.

The titration of Cd_4-α with Cu(I) supported and extended the findings with the holoprotein (Figure 2). The spectrometric

breakpoints of 4 and 6 Cu/mol of MT, the isosbestic point for the first titration process (0–4 Cu/mol of Cd_4-α) and the endpoint of 4 for displacement of Cd by Cu by metal analysis, and the binding of two more Cu(I) ions by the structure clearly indicated the existence of two modes of binding of Cu to MT, Cu_4S_{11} and Cu_6S_{11}.

The conclusions from the metal analyses accompanying the spectrophotometric titrations are consistent with the NMR titration results described in Figure 5, which showed that the entire spectrum of $^{111}Cd_7$-MT 2 from rabbit liver was lost after 7.5 g-atoms of Cu(I) per mol of protein had been added to the sample. According to the NMR results, the successive steps in the replacement of Cd with Cu can be described with approximate stoichiometry as follows:

$$(Cd_4)_\alpha(Cd_3)_\beta\text{-MT} + 3Cu \rightarrow (Cd_4)_\alpha(Cu_3)_\beta\text{-MT} + 3Cd \quad (1)$$

$$(Cd_4)_\alpha(Cu_3)_\beta\text{-MT} + 3.5Cu \rightarrow (Cu_4)_\alpha(Cu_{3-4})_\beta\text{-MT} + 4Cd \quad (2)$$

In the first part of the reaction, shown in eqn. (1), Cu specifically entered the β-domain and formed primarily Cu_3S_9 clusters through a co-operative displacement process along with a small amount of a mixed Cu_2,Cd cluster, represented by the appearance of a new ^{111}Cd resonance at 648 p.p.m. Additional Cu completed the one-for-one selective replacement of Cd from the β-domain. This species was previously observed by Briggs and Armitage [11] and was also seen in the present paper after replacement of Zn by ^{111}Cd in Cu_3Zn_4-MT 2 from calf liver (Figure 6). Thereafter, according to the titration, one more Cu(I) ion may have entered the β-domain before Cu co-operatively displaced Cd from the α-domain cluster to form $(Cu_4)_\alpha$ peak intensity upon addition of the fourth Cu(I) ion. Taken together, the various titration results indicate that the α-domain binds 4 or 6 Cu(I) ions and that the β-domain can accommodate 3 or, perhaps, 4 Cu(I) ions. No titration data clearly revealed the formation of a Cu_6S_9 β-domain.

The possibility that a Cu_4S_9 cluster can form agrees with a recent paper by Pountney et al. [13] showing a breakpoint in the titration of apoMT by Cu(I) at 8 Cu per mol of MT, according to their interpretation each domain forms a Cu_4S_{6-7} cluster, leaving some free thiol ligands in each domain. In the many determinations of sulphydryl concentration in Cu-containing MT samples in this and the accompanying study [1], there was no indication of rapid reaction of a fraction of unbound thiols with 5,5′-dithiobis-(2-nitrobenzoate). Thus, it is hypothesized in the present work that all the sulphydryl groups are involved in clusters of various Cu content.

Examination of native forms of calf liver MT (Cu to Zn ratios of 3 to 4 and 6 to 4) after substitution of Zn by ^{111}Cd showed that Cd was localized almost entirely in the α-domain in a homogeneous Cd_4S_{11} cluster of each protein (Figure 6). By inference, Cu was present primarily in the β-domain as either a Cu_3S_9 or Cu_6S_9 cluster. Additional support for the segregation of Cu into one domain came from two experiments. In the first, Cu(I) was removed from Cu_6,Cd_4-MT with BCS, the protein was subjected to subtilisin, and the products were chromatographed over Sephadex G-75. The Cd remaining bound to the protein was confined to a fragment of MT, consistent with its localization in one of the domains (Figure 7). A similar experiment conducted with Cu_6,Zn_4-MT also supported the segregation of metal ions. This protein was reacted first with NTA to remove Zn selectively from the structure and then with subtilisin to digest protein that had previously bound the Zn ions. The products were separated as above by gel filtration, revealing the presence of a fragment of MT, which bound only Cu and which migrated like an intact domain of the protein (Figure 8). Agreement between this and the ^{111}Cd-NMR experiment suggests that Cd substitution for Zn did not perturb the location of Cu in the protein. So, examination of native, Cd-substituted samples from the present work supports the formation of alternate β-domain Cu(I)–thiolate structures, Cu_3S_9 as well as one with an expanded number of Cu ions, Cu_6S_9.

The stoichiometry of cluster occupancy by Cu shown in this set of experiments with either Cd_7-MT or apoMT differs from previous results of the titration of apoMT with Cu(I) [2–4]. In those studies, apoMT was reacted with limiting amounts of Cu(I) presented anaerobically to the protein as CuCl and then digested with subtilisin to hydrolyse protein that had not folded into proteolytically resistant α- or β-domains. As in the current experiments, Cu localized first in the β-domain, but apparently yielded only a Cu_6S_9 cluster and not initially a Cu_3S_9 species. Then, it entered the α-domain to generate $(Cu_6)_\alpha(Cu_6)_\beta$-MT, whereas $(Cu_4)_\alpha(Cu_{3-4})_\beta$ was observed in the present work. Both steps involved apparent co-operative, all-or-nothing, formation of clusters. The differences in these results may be due to the presence of subtilisin in the latter experiments. It adds to the system a thermodynamic driving force that may favour different stoichiometries of reaction and locations of binding for Cu(I) in the protein.

There is also evidence in the present results for the existence of minor amounts of mixed Cu,Cd clusters in Cu,Cd-MT. Besides the appearance of a new resonance during the titration of $^{111}Cd_7$-MT with Cu(I), the serial reaction of Cu_6,Cd_4-MT with BCS and then subtilisin left a small amount of unreacted Cu,Cd protein, which may have contained heterogeneous metal clusters that were resistant to reaction with BCS as found previously [1]. In addition, the ^{111}Cd spectrum obtained earlier for Cu_3,$^{111}Cd_4$-MT displayed minor ^{111}Cd resonances that apparently represent mixed Cu,Cd species [11].

A final question arises from this work. How is selective localization of Cu in the β-domain achieved kinetically, given that the displacement of Cd by Cu is overwhelmingly favoured in both clusters and that under the conditions used in the present experiments, Cd displacement by Cu(I) occurred rapidly in both domains? In the case of the reaction of Zn_7-MT with Cd, one does not see this selectivity and Cd is kinetically trapped in mixed Cd,Zn clusters in both domains that apparently are not thermodynamically favoured products [8,14]. As in the reaction of Cd with Zn_7-MT, one expects that the initial, random attack of Cu(I) on Cd_7-MT will displace Cd from both clusters.

$$(Cd_4)_\alpha(Cd_3)_\beta\text{-MT} + (j+k)Cu^+ \rightarrow (Cu_j,Cd_{4-j})_\alpha(Cu_kCd_{3-k})_\beta\text{-MT} + nCd^{2+} \quad (3)$$

Then, to account for the observed localization of Cu in the β-domain, it is hypothesized that in contrast with Cd,Zn-MT formed from Zn_7-MT and Cd^{2+}, heterogeneous Cu,Cd clusters in eqn. (3) undergo interprotein rearrangement rapidly enough to achieve the final observed segregation of metals among the two clusters.

The mechanism which accounts for the native distribution of metals in Cd,Zn-MT involves interprotein exchange of metals among homogeneous metal Cd- and Zn-MT [8]. In the present study, such a reaction has been documented for Cd- and Cu-MT species. The results shown in Figure 9 indicate that interprotein metal exchange can occur between rabbit liver $^{111}Cd_7$-MT 2 and Cu_{10}-MT 2, leading to selective and largely co-operative exchange of Cd in the β-domain of one protein with Cu in the α-domain of the other. Products of this reaction are (Cd_4S_{11})-α and Cu–thiolate clusters in the β-domain. Since Cd has a preference for clustering in the α-domain and Cu for binding in the β-domain, interprotein exchange is favoured by the redistribution of both Cd and Cu [3–5].

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