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ABSORPTION, CIRCULAR DICHROISM, MAGNETIC CIRCULAR DICHROISM AND EMISSION STUDY OF RAT KIDNEY Cd,Cu-METALLOTHIONEIN

Martin J. STILLMAN ** and Jadwiga A. SZYMANSKA *,**

Department of Chemistry, University of Western Ontario, London, Ontario N6A 5B7, Canada

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Absorption, circular dichroism (CD), magnetic circular dichroism (MCD) and emission spectra of rat liver and rat kidney cadmium-, zinc- and copper-containing metallothioneins (MT) are reported. The absorption, CD and MCD data of native rat kidney Cd,Cu-MT protein closely resemble data recorded for the rat liver Cd,Zn-MT. This suggests that the major features in all three spectra of the native Cd,Cu-MT are dominated by cadmium-related bands. The CD spectrum of the Cd,Cu-MT recorded at pH 2.7 has the same band envelope that is observed for a Cd,Cu-MT formed in vitro by titration of Cd,Zn-MT with Cu(I), suggesting that the copper occupies the zinc sites in Cd,Cu-MT formed both in vivo and, at low molar ratios, in vitro. Remetallation of the metallothionein from low pH in the presence of both copper and cadmium results in considerably less cadmium bound to the protein than was present in the native sample. It is suggested that this is due to the effect of the distribution of the copper amongst all available binding sites, thus inhibiting cluster formation by the cadmium. Emission spectra are reported for the first time for a cadmium- and copper-containing metallothionein. An emission band at 610 nm is shown to be a sensitive indicator of Cu(I) binding to metallothionein. Both the native Cd,Cu-MT and a Cd,Cu-MT formed in vitro exhibit an excitation spectrum with a band in the copper-thiolate charge-transfer region.

1. Introduction

The protein metallothionein (MT) was first identified as a cadmium- and zinc-containing protein by Margoshes and Vallee [1]. Since that time, metallothioneins from a wide variety of sources have been studied. It is now established that the metallothioneins are characterized by a low molecular weight (generally in the range 6000–10000), a very high fraction of cysteine residues (about a third in most cases) and a metal content of between five and seven atoms per molecule of protein [2].

The most widely studied metallothionein has been Cd,Zn-MT; this is due in part to the ease of induction of the protein in rat and rabbit livers, and in part to the properties of the cadmium ion itself which allows the use of both the radioactive ^{109}Cd isotope and the ultraviolet absorption spectrum for identification purposes during preparation. Considerable spectroscopic data are now available for this protein [3–8]. While it is known that many other metals also induce synthesis of metallothioneins containing metals other than either cadmium or zinc [9], far less spectroscopic data have been reported for these proteins.

In this paper we describe the first optical study of a Cd,Cu-metallothionein formed in vivo. Data presented here also demonstrate the utility of the emission spectrum of the copper-sulfide chromophore in the Cd,Cu-MT as a sensitive indicator of the presence of the copper.

* Permanent address: Department of Toxicological Chemistry, Medical Academy, Lodz, Poland.
** Associated with the Centre for Interdisciplinary Studies in Chemical Physics at the University of Western Ontario.

2. Experimental

Cd,Zn-MT and Cd,Cu-MT were prepared as previously reported [10,11] from the livers (Cd,Zn-MT) and kidneys (Cd,Cu-MT) of rats following induction of the metallothionein with injections of $CdCl_2$. Metal content in the proteins was determined by atomic absorption spectroscopy (Varian 875 in the flame mode) – for Cd,Zn-MT Cd : 2.0, Zn : 1.7 and Cu : 0.3, while for Cd,Cu-MT Cd : 1.6, Zn : 0.2 and Cu : 2.5, where the values are expressed here as mol metal/mol protein. The concentrations of the MT samples were calculated by setting the molar absorption at 250 nm to 57 000 l mol^{-1} cm^{-1} [6,12]; the actual absorption of each sample is also quoted in each figure caption. Absorption spectra were recorded on a Cary 219 spectrometer, CD spectra on a Jasco J5 with Sproul SS20 specifications, and emission spectra were recorded on a Perkin Elmer MPF 4. The emission and excitation spectra were recorded in the ratio mode of the spectrometer. Because the phototube used was a Hamamatsu R928, the visible region response in the emission spectrum is essentially flat. The use of the ratio mode in excitation corrects for loss of intensity of the lamp but not of the monochromator and the analysing photomultiplier tube. We measured the excitation spectrum of quinine sulfate in 0.1 M H_2SO_4 and compared this with its absorption spectrum. The ratio of emission intensity at a fixed wavelength measured for a variable λ_{ex}, divided by the absorptivity at that same wavelength gives an indication of the change in response of the spectrometer throughout any given wavelength range. For the range of interest here, i.e., 225–350 nm, the response was essentially constant when the ratio mode was used with a dilute solution of quinine sulfate and thus an excitation spectrum measured using the ratio mode does give a fair indication of the true excitation spectrum. MCD spectra were recorded on the J5 spectrometer using an Oxford Instruments (U.K.) SM2 magnet operating at 5.5 T to supply the field. The CD spectrometer was calibrated for intensity using d_{10}-camphorsulfonic acid, with $[\theta]/\varepsilon = 226$ [13], and the intensity of the MCD spectrometer was calibrated using an aqueous solution of $CoSO_4$; we obtain $[\theta]_M$ (505 nm) = -61.6 deg. cm^2 $dmol^{-1}$ T^{-1} or $\Delta\varepsilon_{505\ nm} = -1.867 \times 10^{-2}$ l mol^{-1} cm^{-1} T^{-1}. Each CD and MCD spectrum was digitized directly from the amplifier. Each MCD spectrum reported here has had the zero-field CD spectrum subtracted from it.

3. Results and discussion

Spectroscopic data are primarily available only for Cd,Zn-MT from livers or for Cu- and Zn-containing metallothioneins made either in vivo following induction by exposure to copper salts [3,14] or by metal substitution into Cd,Zn-MT in vitro [15]. In this work we have used Cd,Cu-MT that was synthesized in rat kidneys following injections of $CdCl_2$. When rats are exposed to cadmium there is an increase in the levels of copper in the kidneys. This enhancement in the copper concentration is accompanied by an increase in the levels of metallothionein-like proteins in the kidneys [16]; these proteins contain mainly cadmium and copper. No optical data have been previously reported for a Cd,Cu-MT formed in vivo, although results from in vitro titrations with copper(I) salts have been described by Rupp and Weser [15] and the first report of the emission spectrum of a copper metallothionein has appeared [17].

Fig. 1 compares the absorption and CD spectra of Cd,Zn-MT and Cd,Cu-MT. The shoulder at 250 nm in the absorption spectrum of Cd,Zn-MT results from sulfur-to-cadmium charge-transfer transitions and gives rise to a characteristic derivative-shaped envelope in both the CD and MCD spectra [18]. The absorption spectrum of Cd,Cu-MT is somewhat less well resolved, and extends further to the red than that for Cd,Zn-MT. This effect undoubtedly arises from the superposition of the rather broad copper(I) charge-transfer envelope [8,14] over the cadmium charge-transfer bands [8,18,19] and the absence of zinc-related bands near 230 nm. However, the CD (fig. 1) and MCD (solid line in fig. 2) of Cd,Cu-MT resemble much more closely the spectra of Cd,Zn-MT [6,8,19] rather than a Cu-MT (although only CD spectra have been published previously for Cu-containing metallothioneins formed in vivo [14]). The minor disparity in intensities between the two CD spec-

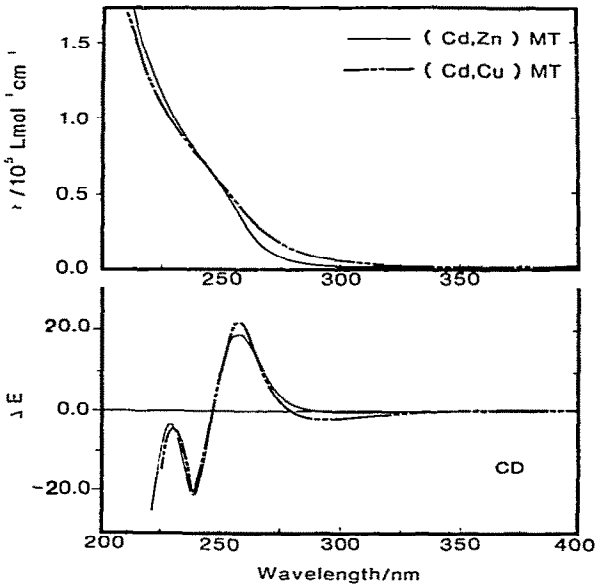

Fig. 1. Absorption and CD spectra of rat liver Cd,Zn-MT (———) ($A_{250} = 0.660$) and rat kidney Cd,Cu-MT (– · · –) ($A_{250} = 0.690$).

tra shown in fig. 1 can be explained by the presence of underlying positive bands in the CD spectrum of Cd,Cu-MT due to the copper thiolate units [3]. We are able to observe these bands in Cd,Cu-MT by reducing the pH to 2.7, this being sufficient to remove the cadmium by competitive protonation of the coordinating thiolates but not so low that the copper is also removed. In a sense this makes a protein that could be called Cu,H-MT. At low pH we do observe (fig. 2) that the CD spectrum is positive between 230 and 275 nm. Thus, the CD spectrum of the cadmium charge-transfer bands in Cd,Cu-MT at neutral pH is, when these positive bands are subtracted, very close to that observed for Cd,Zn-MT. We can speculate that the two metals occupy different clusters within each metallothionein molecule, following the conclusions of Briggs and Armitage [20], and that, therefore, the environment around the cadmium-binding sites in Cd,Cu-MT should be quite similar to that in Cd,Zn-MT, which in turn leads to the similar CD trace for native Cd,Cu-MT.

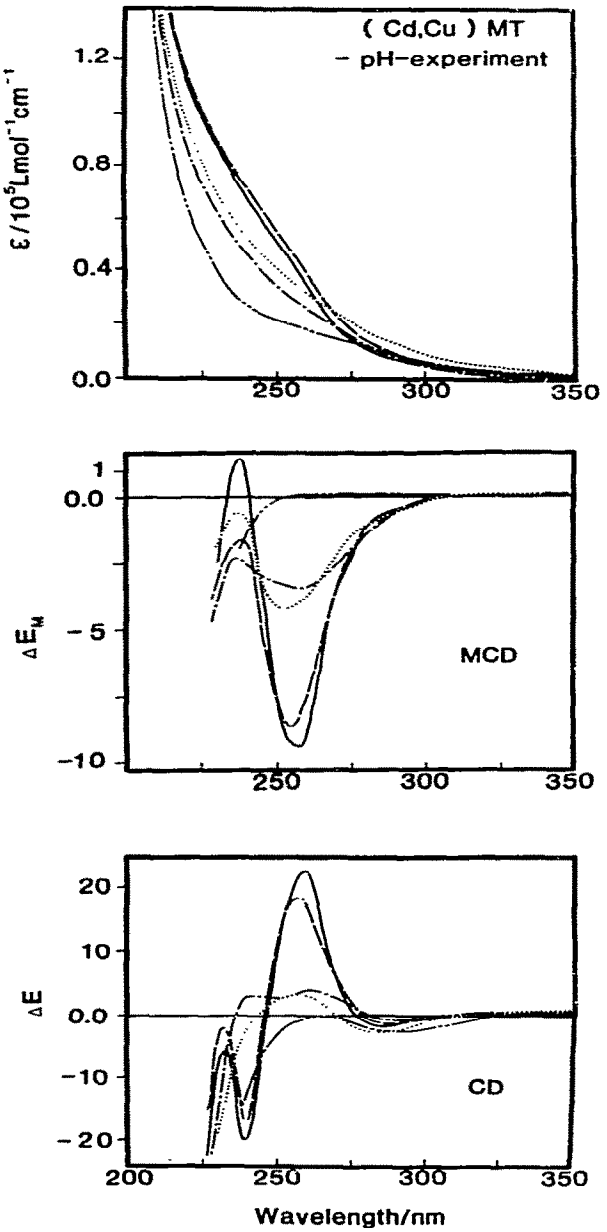

Fig. 2. The pH dependence of the absorption, MCD and CD spectra of Cd,Cu-MT. The pH cycle was 7.1 (———), 5.70 (- - - - - -) 2.7 (– · –), 1.1 (– · · –), then the pH was raised to 7.4 (· · · · · ·). The same solution was used for all spectra: $A_{250} = 0.540$, initially.

In experiments with Cd,Zn-MT from rat liver, crabs and guinea pig livers in which the pH was cycled from 7.6 to 1.0, then back to 7.6, using both optical [12,19] and ^{113}Cd-NMR [21] techniques as probes of changes in the region of the cadmium-binding sites, we have demonstrated that cadmium binds to metal-free MT with a binding site geometry that is very similar to that in the original protein.

Fig. 2 also shows the results of such a pH experiment for the Cd,Cu-MT. Of special interest in these data is the way in which the CD and MCD envelopes change in the 250 nm wavelength region as the pH is lowered and then raised. As the pH drops from 7.1 to 1.1 we observe that the intensity at 250 nm in all three spectra (absorption, MCD and CD) diminishes. However, compared with this same pH span for Cd,Zn-MT, we find that there is still considerable intensity at pH 2.7. By pH 1, the spectrum shows that all the metal has dissociated from the protein. The pH is now raised to 7.4. Unlike Cd,Zn-MT [12,19,21], we find that the protein does not rebind the metal completely. While the absorption spectrum of the protein at pH 7.4 shows very poor resolution and gives little indication of any specific binding (there are apparently no resolved bands near 250 nm to suggest metal chelation by the protein), the MCD does show quite clearly that about 40% of the total number of cadmium ions are bound to sites of the same, approximately tetrahedral geometry [6] as in the native protein. It is now possible to reconcile the lack of resolution in the absorption spectrum because the 250 nm charge-transfer band should have only 40% of the intensity found for the native protein, and when this is added to the broad tail of the metal-free protein at pH 7 we obtain an absorption spectrum with little resolved detail.

The CD spectra in fig. 2 show in considerable detail that the protein environment around the Cd- and Cu-thiolate groups changes as the pH is changed. The CD intensity arises from mixing of the chirality of the coordinating cysteine residues into the absorption band by the charge-transfer transitions involving the sulfurs and the metal ions [8]. Thus, the CD intensity reflects the chiral environment around the metal-binding sites. Quite unlike the data recorded for pH titrations of Cd,Zn-MT, the CD spectra for Cd,Cu-MT (fig. 2) do not follow a straightforward reduction in intensity as the pH is lowered. Instead, at intermediate pH values, where the cadmium is known to have been dissociated [8], a new envelope is observed that is characterized by a positive band at 240 nm (the trace recorded at pH 2.7 in fig. 2). This feature has also been reported by Rupp et al. [3] in the pH titration of a copper-containing metallothionein that had been formed by adding a single aliquot of Cu(I) to Cd,Zn-MT. Very recently, we have been able to demonstrate (A.Y.C. Law and M.J. Stillman, unpublished results) the formation of the same CD spectral feature during a detailed titration of rat liver Cd,Zn-MT with Cu(I) at neutral pH. The loss of an isosbestic point and the molar ratio of copper to protein indicated that while only the zinc sites were fully occupied by copper, sufficient excess copper had been added to dissociate the cadmium cluster; this dramatically diminished the CD intensity from the S → Cd charge-transfer bands. Thus, this species is also a model for Cd,Cu-MT at pH 2.7. It is quite surprising that these three protein samples, one metallated in vivo, the other two in vitro, exhibit such similar CD spectra. In light of the two-cluster model for the MT metal-binding sites [5,7] these results suggest that the copper bound in vivo occupies the same cluster as the copper that was bound in vitro where it undoubtedly initially displaced the zinc. This suggests that in Cd,Cu-MT, the copper occupies the three-metal cluster known as cluster B [7].

In the case of the remetallated Cd,Cu-MT, the CD spectrum changes significantly when it is compared to the spectrum of the native protein, which suggests that the protein structure near the cadmium is also different. Because, in the native protein, there are both cadmium and copper ions present, it is likely that as the pH is raised the copper binds to the metal-free MT at a much lower pH than the cadmium [4]. These data suggest that the copper binds to the metal-free MT in sites that are different from those occupied in the native protein. As the pH is raised through the range where cadmium binds only 40% of the original sites now remain for the cadmium and these sites are not quite the same as those in the native

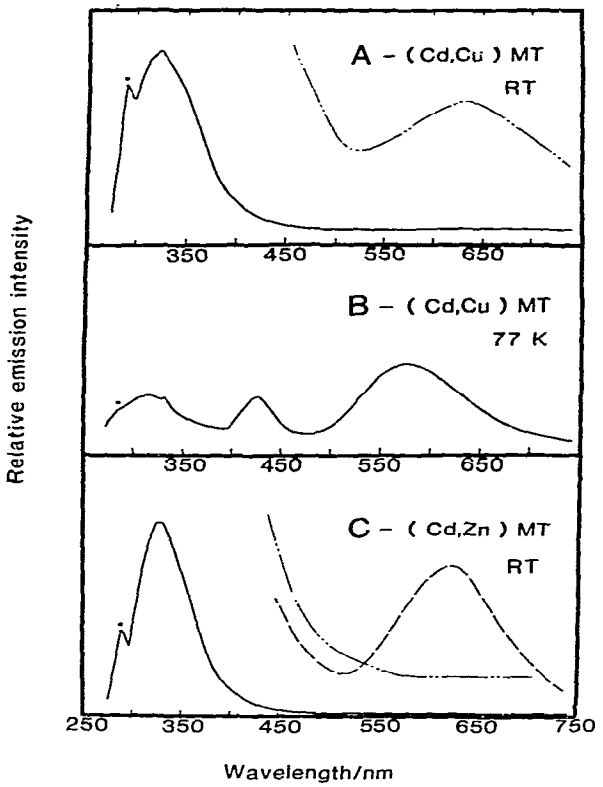

Fig. 3. Emission spectra (uncorrected) for Cd,Cu-MT and Cd,Zn-MT. (A) Cd,Cu-MT; $\lambda_{ex} = 260$ nm for both spectra: (– –) sensitivity 100× greater; both spectra recorded at room temperature; $A_{250} = 0.690$. (B) Cd,Cu-MT; $\lambda_{ex} = 260$ nm; the spectrum was recorded at 77 K using an aqueous-glycerol glass. (C) Cd,Zn-MT; $\lambda_{ex} = 260$ nm for all three spectra: $A_{250} = 0.660$; (–·–·–) sensitivity 100× greater; (— — —) the same sample of Cd,Zn-MT with 3.25 mol equiv. Cu(I) added; the spectra were recorded at room temperature and Cu(I) was added under a nitrogen atmosphere. The band identified with an asterisk near 290 nm in each emission spectrum is a Raman band from the solvent.

protein, although the MCD data clearly shows that they still have an approximately tetrahedral geometry. It is probable that cluster formation for the cadmium is inhibited by the distribution of copper in sites throughout the protein.

The emission spectra for the Cd,Cu-MT and Cd,Zn-MT proteins recorded using excitation at 260 nm are shown in Fig. 3A and C, respectively. At room temperature both proteins display a single, broad band centered at 320 nm. However, when a sensitivity 100-times greater is employed, a new, very broad band is observed near 610 nm for the Cd,Cu-MT sample (dashed line in fig. 3A). A similar band is not seen in the Cd,Zn-MT spectrum recorded under comparable conditions (dashed line in fig. 3C). λ_{max} for this red emission is invariant with respect to the exciting wavelength in the range 240–310 nm. This red emission resembles the 580 nm bands observed for a copper-containing metallothionein isolated from *Neurospora crassa* by Beltramini and Lerch [17] and for a variety of copper complexes reported by Anglin et al. [22]. Spectra recorded from frozen glasses at 77 K (using a 50:50 (v/v) aqueous glycerol solvent to aid glassing) show that the 320 nm band is a fluorescence band; a new, but weak, phosphorescence intensity at 440 nm, which is observed for both proteins, can be attributed to the solvent. Because of the very high purity of Cd,Zn-MT, and the close alignment of this phosphorescent band in the protein sample with a band observed in a solvent blank, we feel that the fluorescence at 320 nm arises from the cysteine residues in the metallothionein rather than from an aromatic amino acid such as tryptophan or tyrosine. A similar band is observed for Hg,Cu-MT [23] and also for Cd,Zn-MT isolated from horse kidney and rabbit liver. (No contribution from tryptophan or tyrosine is observed in either the absorption, CD or MCD spectra and no aromatic amino acids are detected by amino acid analysis.)

Excitation spectra were obtained by monitoring the emission intensities at 320 and 580 nm using the ratio mode of the spectrometer (fig. 4). Both proteins exhibit an apparent excitation spectrum, recorded using 320 nm excitation, that consists of two bands: one near 230 nm and the other at 280 nm. However, the apparent excitation spectrum obtained by monitoring the emission at 580 nm observed for the Cd,Cu-MT is quite different (fig. 4C). Because these samples absorb very strongly below 260 nm, the apparent excitation spectra must be corrected for the wavelength dependence of the loss of intensity of the exciting light as the

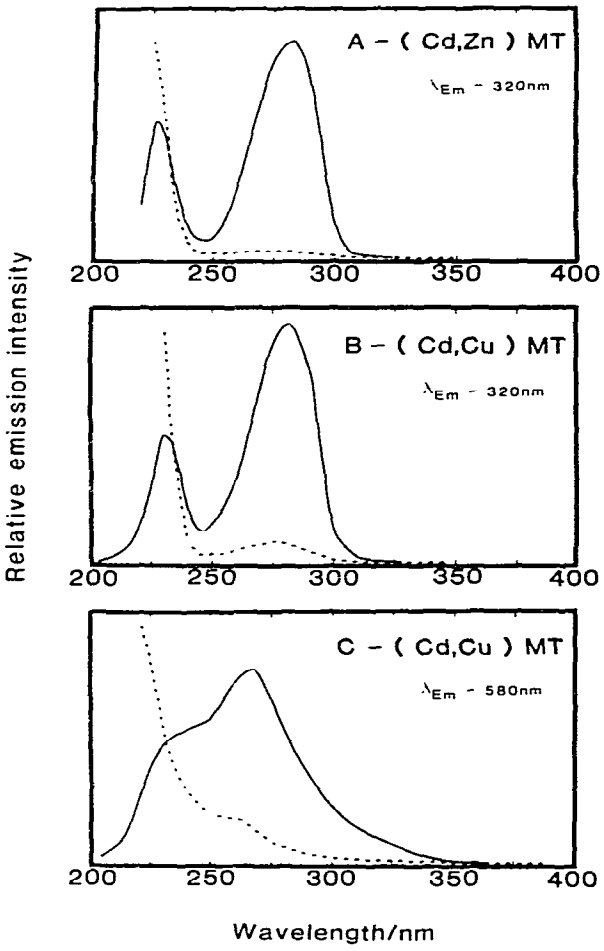

Fig. 4. Excitation spectra for Cd,Zn-MT and Cd,Cu-MT. (A) Cd,Zn-MT; $\lambda_{em} = 320$ nm. (B) Cd,Cu-MT; $\lambda_{em} = 320$ nm. (C) Cd,Cu-MT; $\lambda_{em} = 580$ nm. (———) Apparent excitation spectrum, (- - - - - -) corrected excitation spectrum, see text for details.

light passes through the cuvette. This was accomplished by normalizing the excitation spectrum with the transmission spectrum. The lack of intensity at 250 nm in the corrected excitation spectra shown in fig. 4A and B suggests that the cadmium-thiolate chromophores do not contribute to the emission intensity recorded at 320 nm. However, for Cd,Cu-MT there is an additional band in the corrected excitation spectrum when the red band is monitored at 580 nm. This strongly suggests that the copper-sulfide chromophore does contribute significant intensity to this band.

We tested the origin of the 610 nm band in the Cd,Cu-MT emission spectrum by adding aliquots of copper(I) to a solution of native Cd,Zn-MT. As the intensity of the zinc- and cadmium-related charge-transfer bands in the ultraviolet absorption spectrum diminished, a new band appeared at 610 nm in the emission spectrum (fig. 3C). This band gave an excitation spectrum that closely resembled that of the native Cd,Cu-MT shown in fig. 4C. By this means we can compare the spectra of two copper-containing proteins: in one the copper was bound in vivo while in the other the copper was bound in vitro. The similarities between both the emission and excitation spectra provide good evidence that the 610 nm band does arise from copper bound to the metallothionein.

We summarize our findings as follows.

(i) The absorption, CD and MCD spectra of Cd,Cu-MT are dominated by cadmium-thiolate transitions and closely resemble the corresponding spectra of Cd,Zn-MT.

(ii) The band at 610 nm in the emission spectrum provides a sensitive indicator of copper(I) binding for both in vivo- and in vitro-formed Cd,Cu-MT. The excitation spectrum shows that this band arises from the copper-thiolate chromophore in the protein.

(iii) Comparison between the CD spectrum of Cd,Cu-MT recorded at pH 2.7 and the CD spectra recorded for Cu-MT formed in vitro indicates that the copper is bound to the zinc sites in the native Cd,Cu-MT.

(iv) In remetallation reactions from low pH, copper competes with cadmium for all possible sites in the protein. This suggests that the reduced number of cadmium ions that bind to the protein arises from the lack of cluster formation.

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