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Isolation and Characterization of Metallothionein from Guinea Pig Liver

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Abstract

The amino acid composition, and the absorption, circular dichroism (CD) and magnetic circular dichroism spectra of a metalloprotein induced in the livers of guinea pigs by the injection of $CdCl_2$ are reported. The amino acid composition of this protein closely resembles that of rat liver metallothionein (MT). We show that this protein has spectroscopic properties that closely follow the behaviour previously reported for several other cadmium-containing metallothioneins in its spectral response to changes in pH, and to the addition of cadmium and copper(I). Dramatic changes are observed in the CD spectrum during the addition of copper(I); it is suggested that these changes are the result of the formation of a mixed Cu(I)/Cd(II) cluster that forms in the α domain once the β domain has been saturated with Cu(I). These results are of particular importance in the characterization of this protein as belonging to the metallothionein class of proteins, as spectral changes of this type are directly related to the displacement of Cd^{2+} and Zn^{2+} from the two, thiolate-cluster binding sites that are amongst the unique properties of mammalian metallothioneins. It is demonstrated that the CD spectrum provides a sensitive indicator of the presence of these special metal binding sites by indicating changes in the binding geometry and stoichiometry in response to an incoming metal. These results indicate that the guinea pig liver metallothionein induced by injections of $CdCl_2$ uses the same α and β type of clusters for cadmium binding as rat liver Cd, Zn–MT, even though there are minor differences in the amino acid composition between the guinea pig and rat liver proteins.

Introduction

The exposure of animals to certain B group metal ions results in the induced synthesis of the protein metallothionein in a variety of tissues; particularly high concentrations of the protein are found in the kidneys and livers [1]. Other metallothioneins have since been identified from a variety of animals and organisms. Besides Cd^{2+}, metal ions such as Zn^{2+}, Hg^{2+}, Cu^{2+}, Au^{3+}, and Bi^{3+} have also been shown to induce synthesis of metallothionein [2]. Depending on the metal ion used for induction, the protein isolated can contain a mixture of metals. Thus Cd^{2+} and Zn^{2+} are found in the protein if Cd^{2+} is used as an inducer and the protein is isolated from the liver, but Cd^{2+} and Cu^{+} are found in MT isolated from the kidneys of the same animal [3]. The widespread occurrence of metallothioneins has led to diverse views regarding possible biological roles in the metabolism, storage, detoxification and homeostasis of metals [4].

The most extensive studies to date have been on cadmium- and zinc-containing metallothioneins obtained from the livers of rats, rabbits and chickens [1, 2]. Optical spectroscopy has provided much information on the relative values of the binding constants for the range of metals that bind to metallothionein *in vitro*. The UV absorption spectrum of many metal-containing MTs is dominated at wavelengths greater than 230 nm by charge transfer intensity from transitions between the thiolate groups and the bound metal ions; for Cd-containing metallothioneins a shoulder at 250 nm is assigned as a $S \rightarrow Cd$ charge transfer band [5]. Analysis of ^{113}Cd NMR data has suggested that in the mammalian Cd- and Zn-containing protein the metals are bound in two clusters, named α and β, which can bind four Cd^{2+} and three Zn^{2+} atoms, respectively [6–8], while in the *Scylla serrata* crab Cd, Zn–MT there are two, β-type, three-metal clusters [9].

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However, specific details on the binding of metals other than Cd^{2+}, and by implication, also Zn^{2+}, are not well known. Optical spectroscopy does provide a sensitive indicator of metal binding when the intensity and positions of metal-related bands can be observed. Although the absorption and magnetic circular dichroism (MCD) data for metallothionein from rat liver and the *Scylla serrata* crab are quite similar, the CD spectra in the charge transfer region of the spectrum of these two proteins are very different [10]. Thus the CD spectrum provides a useful criterion for the comparison of the conformation of the amino acid residues around the metal in different metallothioneins. MCD spectroscopy can be used to probe the geometry of the coordinating ligands that bind the metal. Cd-containing metallothioneins from several different species have been shown to exhibit the same, unique CD and MCD spectra [1, 2, 10–12].

In this work, we describe the isolation and purification of a Cd-containing protein induced in guinea pig livers by injections of $CdCl_2$. The amino acid composition and the characterization of this protein by UV absorption, CD and MCD techniques are presented as compelling evidence that this protein belongs to the metallothionein class. Metal binding experiments with Cd^{2+} and Cu^{+} are described that provide a unique criterion for the confirmation of the presence of metal binding in the clustered binding sites that are characteristic of mammalian metallothioneins.

Materials and Methods

Adult, female English short-hair Albino strain guinea pigs (Connaught Laboratory, Toronto, Ont.) were injected subcutaneously with 1 mg Cd/kg, on alternate days over a two week period. The total injected dose was 8 mg Cd/kg. Two days after the last injection, the animals were sacrificed by exsanguination and livers were homogenized in 0.25 M sucrose, 0.1 M tris-HCl buffer pH 8.0. The metallothionein was isolated by methods described elsewhere [13] and further separated on DEAE-cellulose columns. Metallothionein-containing fractions eluted from DEAE-cellulose columns were designated as GP-MT **1** and **2**. These two fractions were pooled separately and freeze dried. The purity of these proteins was determined by amino acid analysis and polyacrylamide gel electrophoresis. Metal content was estimated by atomic absorption spectrophotometry. Protein concentrations were estimated using analysis for SH groups with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in guanidine hydrochloride. Spectroscopic measurements were made as previously reported [11, 14] using a Jasco J5 CD spectrometer modified to Sproul SS20 specifications or a Jasco J500 CD spectrometer. The optical data were digitized directly from the spectrometer.

Results and Discussion

Guinea pig liver metallothionein (GP-MT) can be separated into two distinct isoforms on gel affinity columns, each exhibits a single band after polyacrylamide gel electrophoresis. These two isoproteins, GP-MT **1** and GP-MT **2**, are heat stable, with similar molecular weight, charge and metal content, containing, on analysis, approximately 4 g atoms Cd^{2+} and 2 g atoms Zn^{2+} per mol. However, the molar ratio of Cd^{2+} to Zn^{2+} does depend on the time delay between the last injection and sacrifice. Amino acid analysis reveals that both GP-MT **1** and **2** have a high cysteine content with no aromatic residues, Table I. Unlike the majority of other mammalian MTs, GP-MT **2** contains at least one residue of arginine, while GP-MT **1** has about 2% leucine. While arginine is not commonly found in metallothionein [2], the amino acid analysis of horse kidney [15] and carp kidney [16] also shows the presence of one residue of arginine. These are the only significant differences in the amino acid compositions of GP-MT **1** and **2** when compared to the same isoproteins from rat liver (Table I). It should be noted that there are considerable variations in the amino acid compositions for metallothioneins reported from different laboratories.

Although the specific differences in the amino acid composition reported here may be characteristic

TABLE I. Amino Acid Composition of Metallothionein **1** and **2** from Rat and Guinea Pig Livers

Amino acid	% of total residues			
	Rat liver		Guinea pid liver[a]	
	1	**2**	**1**	**2**
Lysine	10.6	12.7	11.3	11.0
Arginine	–	–	0.0	1.6
Histidine	–	–	–	–
Aspartic acid	6.5	7.0	5.6	6.9
Threonine	4.3	3.0	6.0	3.3
Serine	8.9	9.3	9.7	8.9
Glutamic acid	3.8	7.2	6.5	5.8
Proline	7.5	11.1	8.3	10.4
Glycine	9.2	6.5	8.5	6.3
Alanine	5.6	8.0	8.7	9.3
Cysteine[b]	37.2	29.8	28.3	31.2
Valine	3.3	1.8	3.6	3.3
Methionine	1.3	1.6	1.6	1.9
Isoleucine	1.5	1.3	0.2	0.0

[a]Guinea Pig liver MT **1** and **2** were analysed in 3 separate samples from different animals and the mean value is shown. [b]Measured as cysteic acid. The samples were hydrolyzed in 6 N HCl for 3 h after performic acid oxidation and the amino acid analysis was carried out in a Beckman amino acid analyser.

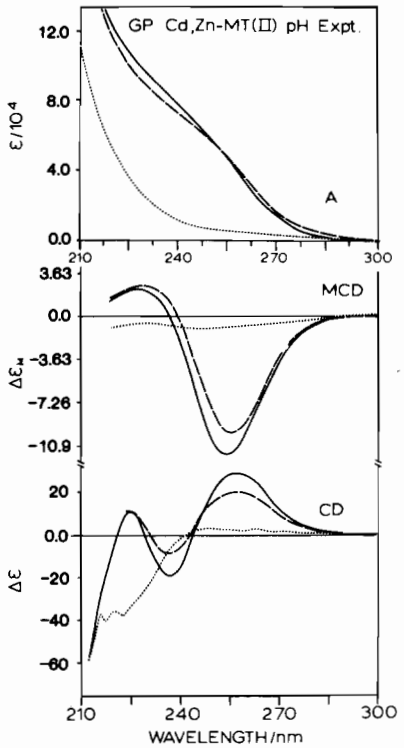

Fig. 1. Absorption (A), MCD and CD spectra of GP Cd, Zn–MT **2**. Initial spectra are of the native protein at pH 7.8, $A_{250\,nm} = 0.42$, (——); spectra at pH 2.3 (· · ·); spectra after the pH had been raised from 2.3 to 7.0 (- - -).

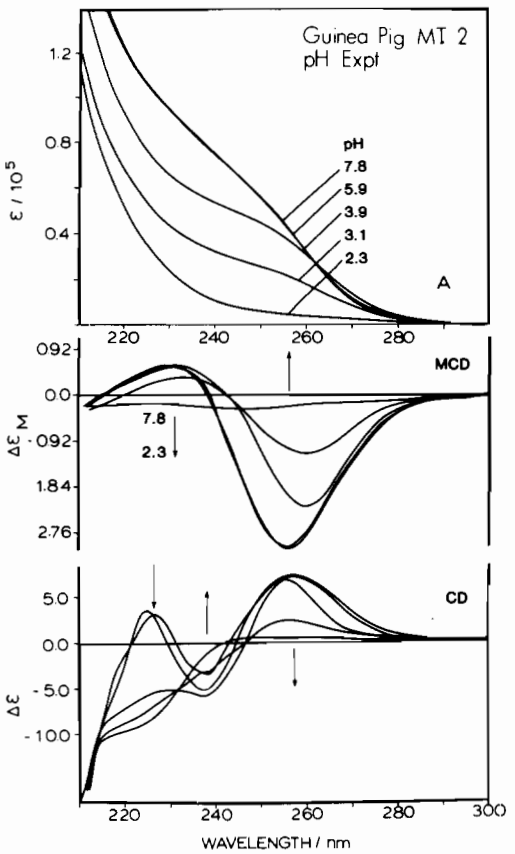

Fig. 2. The effect of pH on the absorption (A), MCD and CD spectra of GP Cd, Zn–MT **2**. The pH of the solution in the cuvette was reduced from 7.8 by adding ul aliquots of concentrated HCl.

of all MT isolated from guinea pig livers, the spectroscopic properties of the GP-MT determined in this work suggests that the metal binding sites are largely unaffected. This seems to be the general case in view of the consistency in metal binding properties that have been obtained from a variety of metallothioneins. However, it may be that the resolution of the spectroscopic techniques can only now be tested as isolation techniques using HPLC allow for the selection of completely homogeneous protein. The full spectroscopic analysis presented here, which includes comparison of the absorption, CD and MCD spectra in Fig. 1, the pH titration in Fig. 2 and, especially, the Cd^{2+} loading experiments shown in Fig. 3, with data previously published, shows that these amino acid differences do not significantly affect the binding sites in the protein. The Cu^{+} loading experiments shown in Fig. 4 provide an even more sensitive indicator of metal binding as the Cu^{+} selectively displaces the β-bound Zn^{2+} and then forms the new complex with the α-bound Cd^{2+}, such data are clearly of value when used with future studies on newly-isolated, cadmium-containing proteins.

The absorption spectra of both GP-MT **1** and GP-MT **2**, Fig. 1, are the same and show the characteristic 250 nm shoulder which indicates the presence of the S → Cd charge transfer band [11, 12]. As shown in Cd–thiolate model compound studies [11, 17, 18], when Cd^{2+} is bound with a tetrahedral geometry a distorted, derivative-shaped MCD signal is observed under the absorption band. The observation of the same CD and MCD spectra as observed for native rat liver and horse kidney Cd, Zn–MT [11, 18–21] confirms the presence of Cd^{2+} in binding sites similar to metallothioneins from other mammalian species.

The effect of pH on the protein spectra has been described previously for chicken and rat liver metallothioneins [12, 21]. The specific features of the data recorded at pH 2.3, Fig. 2, the absence of absorbance at 250 nm and the flat CD spectrum, with the negative tail in the 220 nm region, are characteristics of the metallothionein's peptide chain [21–25]. Following the experiment shown in Fig. 2 the pH of each solution was raised to near 7. In each case the absorption, CD and MCD intensities returned to almost the same values as those observed for the native protein. Only the spectra for the remetallation from the lowest pH used (2.3) are shown in Fig. 1. The red shift of the MCD band provides evidence that

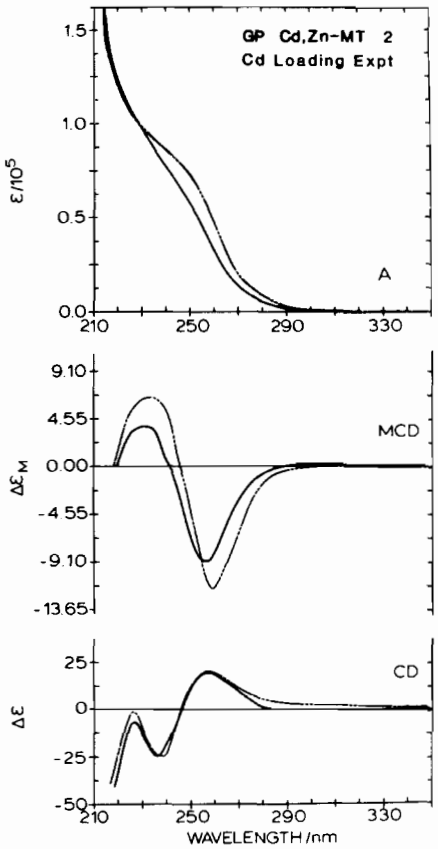

Fig. 3. The effect of adding Cd^{2+} to GP Cd, Zn–MT **2** on the absorption (A), MCD and CD spectra. Native protein at pH 8.5 ($A_{250\,nm} = 0.57$) (——); with 24.5 mol equivalents of Cd^{2+} added (– ·· –).

only the Cd^{2+} binds to the metal-free MT. The 3 or 4 nm red shift of the MCD band at 250 nm following acidification to pH 3.9 or remetallation with cadmium (Figs. 1 and 2) also appears to be a property of Cd, Zn–metallothioneins [21] and observation of this spectral effect with this protein provides evidence for the metallothionein classification. The reappearance of the CD and MCD envelopes does, however, confirm that the Cd^{2+} is rebound to the protein in a similar manner to the native protein [21, 24].

The effect of Cd^{2+} loading on this protein provides evidence for the type of binding used for both the Zn^{2+} and Cd^{2+}. As the binding affinity of Cd^{2+} for the thiolate groups in MT is higher than for Zn^{2+} [1, 5, 25], the addition of Cd^{2+} to the native protein will displace the endogenous, protein-bound Zn^{2+}. In Fig. 3, the increase in the resolution of the 250 nm shoulder and the appearance of a more symmetrical MCD signal indicates that the zinc has been displaced by the cadmium. Interestingly, as with other metallothioneins, we find remarkably little change in the CD spectrum even when an extra 3 mol equivalents of cadmium are bound to the protein. With rat liver Cd, Zn–MT it has been possible to identify both a Cd_7–MT and a Cd_{10}–MT species [26], both of which show remarkably similar CD spectra, with the exception of a band near 228 nm. With the addition of the guinea pig MT data, we can be more certain that the lack of a Beer's Law relationship between the CD intensity at 255 nm and the number of Cd^{2+} ions bound to the β site is a characteristic property of cadmium binding to MT.

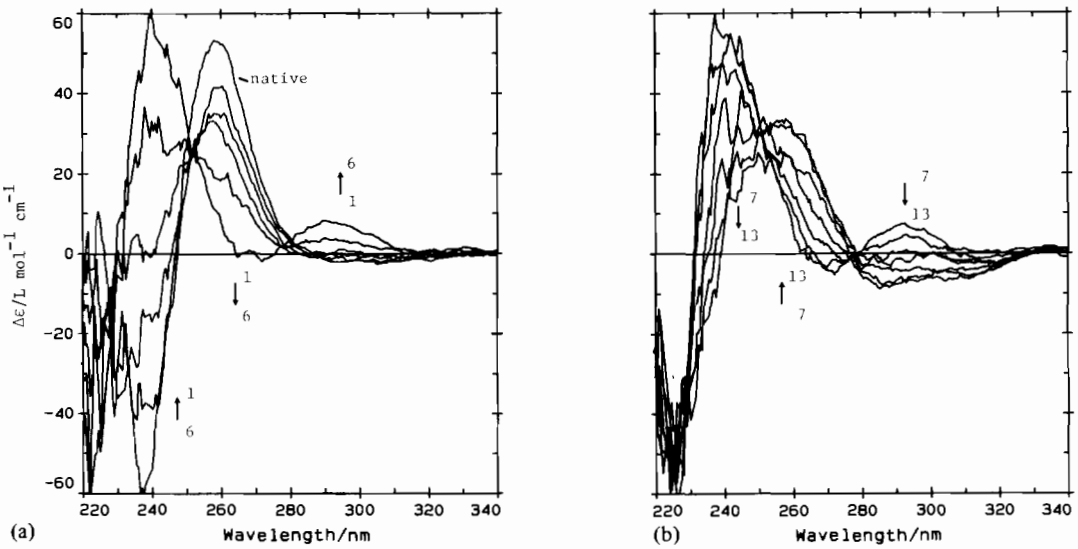

Fig. 4. The effect of adding Cu^{+} to GP Cd, Zn–MT **2** on absorption and CD spectra. The metal content of this solution was: Cd^{2+} = 6.8 mol equivalents and Zn^{2+} = 0.2 mol equivalents; the cuvette contained 10.0×10^{-9} mol/ml protein. Mole equivalents of Cu^{+} added to the cuvette were: (1) 0, (2) 2.0, (3) 4.0, (4) 6.0, (5) 8.0, (6) 10.0, (7) 11.0, (8) 12.0, (9) 13.0, (10) 14.0, (11) 16.0, (12) 18.0, (13) 20.0. (a) shows data up to 10.0 mol equivalents Cu^{+}; (b) shows the last part of the titration from 11.0 to 20.0 mol equivalents Cu^{+}.

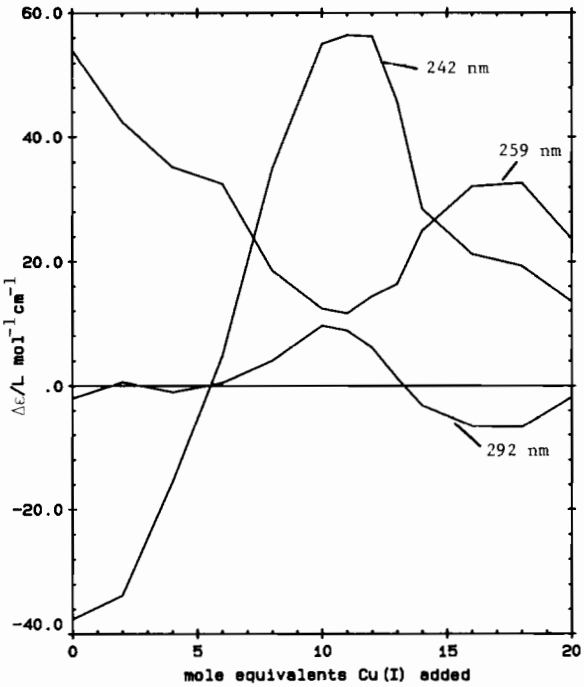

Fig. 5. The change in the CD intensity at three different wavelengths (242 nm, 259 nm and 292 nm) as aliquots of Cu^+ were added to the guinea pig metallothionein.

We have found previously with rat liver metallothionein [26] and with rat liver alpha fragment [27], that Cu^+ binding takes place in several distinct steps. (We note that Cu^{2+} does not bind *in vitro* in this same manner [1, 2]). The reaction that was previously reported [27] applied to cadmium-saturated α fragment. In this present study we have investigated the effects of adding Cu^+ to a metallothionein where both α and β domains are filled with metal ions that are known to be replaced by Cu^+. It is significant that we have now been able to obtain a set of data from the titration of this new GP Cd, Zn–MT protein that closely resembles the set reported for the α fragment alone [27]. Figs. 4a and 4b show the four distinct stages of Cu^+ binding monitored using the CD spectra recorded during a titration of GP Cd, Zn–MT 2 with the $[Cu(CH_3CN)_4]^+$ complex.

Initial addition of up to 4.0 mol equivalents of Cu^+ (lines 1–3) resulted in a gradual, isosbestic (isosbestic point at 248 nm) decrease in the CD signal intensity due to the Cd^{2+}. Figure 5 depicts the changes in CD signal during the titration for several different wavelengths. The plateau observed for the 259 and 292 nm bands between the addition of 4 and 6 mol equivalents Cu^+ tends to suggest that saturation of a specific Cu^+ binding site occurred near these molar ratios. The second stage in the reaction is marked by a red shift in the isosbestic point to 252 nm. From 4.0 to 10.0 mol equivalents, a new spectrum takes over (lines 3–6 in Fig. 4a); the saturation between 10 and 12 mol equivalents is clear at all three wavelengths shown in Fig. 5. This spectrum is characterized by positive bands at 242 nm and 292 nm. The third stage of the titration (lines 8–12 in Fig. 4b) is characterized by a shift of the 243 nm band to longer wavelengths (258 nm), as well as an inversion in the 292 nm band intensity from positive to negative. Two, new negative bands form, at 286 and at 311 nm. Finally, the beginning of the fourth stage is marked by the non-isosbestic decrease in the 258 nm intensity and an increase in intensity near 293 nm (line 13).

The spectral changes shown in Fig. 4a and b are very similar to those obtained with rat liver Cd_4–alpha fragment [27]. From the intensity data at 259 and 292 nm shown in Fig. 5, it can be seen that the CD signal changes become significant only after about 6 mol equivalents Cu^+ has been added. This suggests that the first stage of the titration involves the filling of the β domain to give a stoichiometry of Cd_4^{α}, Cu_6^{β}–MT. This arrangement is supported by the work of Nielson and Winge [28] and Nielson *et al.* [29], in which six Cu^+ were found in the β domain following proteolysis of the protein. Addition of Cu^+ beyond this point results in the isosbestic formation of a new CD spectrum with positive bands at 242 and 292 nm, and a negative band near 271 nm. A similar CD spectrum is also obtained for Cd_4–alpha fragment following the addition of 6 mol equivalents Cu^+ [27]. MCD data from previous Cu^+ binding experiments with rat liver Cd, Zn–MT [26] and the alpha fragment [27] indicate that the Cd^{2+} is no longer bound with a symmetric geometry. We suggest that these spectral results can occur when the bridging sulfurs in the Cd_4-α cluster rearrange to allow the binding of the incoming Cu^+. In the new arrangement, there may still be a 4-coordinate Cd^{2+}-cluster but it may involve only 2 or 3 sulfurs, together with a ligand other than sulfur, for example an oxygen. That the CD spectrum shown as line 6 in Fig. 4a requires the presence of Cd^{2+} is supported by the following evidence: (i) no such spectra are found when Zn^{α}, Zn^{β}–MT is studied, unless Cd^{2+} is added before the α cluster becomes saturated with Cu^+ [28] and, (ii) metal analysis following dialysis of samples of GP Cd, Zn–MT and alpha fragment after the Cu^+ titration at the stage that the new CD spectrum was displayed, indicated a 1:1 ratio for Cd:Cu [27]. We should note, however, that this stoichiometry (of 1:1) does not mean that the stoichiometry in the metallothionein is 1:1 before dialysis, because we are sure that during the dialysis the complex falls apart and the metals rearrange. However, clearly, Cd^{2+} is present together with the Cu^+. We suggest that the Cu^+ is bound in the α domain in similar fashion to

the β domain, such that 9 sulfurs are used in a Cu_6S_9 cluster, the Cd^{2+} then can bind using the last two sulfurs, with bridging to the Cu^+ cluster, together with neighbouring oxygens [28]. An example of a mixed-metal cluster involving both mono- and divalent metals has been reported by Birker *et al.* [31].

It is not yet clear whether there are any specific differences in Cu^+ binding between the α and β domains. The CD spectra recorded at the 12 mol equivalents point for GP Cd, Zn–MT look almost identical with those recorded for Cd_4-α fragment at the 6 mol equivalents point. We have found that binding experiments seem to give cleaner isosbestic points when Zn–MT has been used as the starting material rather than a mixed-metal Cd, Zn–MT protein, but we have not yet been able to identify a significant reason for this [28]. However, the data in this paper do represent the first description of metal binding for a mixed-metal metallothionein in which during the titration a mixture of metals are clearly bound in the α domain. It is particularly significant that the Cd^{2+} is retained in the protein even in the presence of so much Cu^+. The only similar, previous report was from emission intensity quenching experiments carried out with $HgCl_2$ on the copper-containing *Neurospora crassa* protein by Beltramini and Lerch [32]. In this work, these authors characterized the binding of two Hg^{2+} ions to free SH groups in the presence of Cu^+. However, the metal binding properties of *N. crassa* are quite different when compared with mammalian metallothioneins. We have now completed discussion concerning the second stage of the titration which we believe has formed the species with an ideal stoichiometry of Cu_{12}, Cd_4–MT, where there are six Cu in the β domain and a mixed Cu_6, Cd_4-α cluster.

Finally, there are two further steps in the Cu^+ reaction. Addition of Cu^+ to the guinea pig MT quenches the new CD spectrum, as Cu^+-saturated metallothionein is formed, Cu_{18}–MT. The saturation point is reached with 18 mol equivalents Cu^+ and this spectrum (line 12 in Fig. 4b) closely resembles that obtained in an *in vitro* titration of Cu^+ with chicken MT [25, 33]. Addition of aliquots of Cu^+ in excess of 18 mol equivalents result in a decrease in the signal at 257 nm and a non-isosbestic increase in CD intensity near 293 nm which we associate with oxidation of the protein.

Conclusions

The biochemical characterization of a protein as a metallothionein is based on the low molecular weight, high cysteine content and group IIB metal binding properties. The results obtained in this work, using amino acid analysis, and absorption, CD and MCD spectroscopies demonstrate conclusively that the protein isolated from the livers of Cd-exposed guinea pigs can be classified as a metallothionein. Of similar importance to the biochemical properties are the specific metal binding properties. The physical methods used in this study allow us to show that the cadmium in this GP protein is bound in a clustered manner and that the binding site behaves like other, well-described metallothioneins with respect to changes in pH and metal substitution. Thus the protein also exhibits the definitive spectral characteristics of the Cd- and Zn-containing rat liver metallothionein, including, perhaps most significantly, the complex changes in the CD spectrum observed as Cu^+ first displaces the Zn^{2+}, then forms a mixed Cu/Cd cluster and, finally, displaces the Cd^{2+} to form a saturated Cu_{18}–MT species.

Acknowledgements

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