Characterization of calf liver Cu,Zn-metallothionein: naturally variable Cu and Zn stoichiometries

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Cu,Zn-metallothioneins were purified from bovine calf liver in order to examine the stoichiometry of metal binding to the protein. Copper and zinc analyses were carried out by atomic absorption spectrophotometry. Consistent quantitative thiolate analyses were obtained spectrophotometrically with Ellman's reagent and amperometrically with phenylmercuric acetate. These were used to define protein concentration. A complementary method to assess the sum of the thiol and Cu(I) content of metallothionein involved titration of the reducing equivalents of the protein with ferricyanide. The stoichiometry of reaction was consistent with the oxidation of all the sulphhydryl groups to disulphides and all of the bound Cu from the cuprous

to the cupric oxidation state. According to these methods, total numbers of zinc plus copper ions bound to metallothionein isolated from a number of calf livers centred on about 7, 10–12, or 15 g-atoms of metal per mol of protein. The reactivity of ferricyanide and 4,7-phenylsulphonyl-2,9-dimethyl-1,10-phenanthroline (BCS) with Cu,Zn-metallothioneins of various metal ratios was assessed. Zinc metallothionein reacted almost entirely in two slow steps with ferricyanide. As the Cu content of the protein increased, the fraction of reaction occurring in the time of mixing increased in parallel. BCS was able to remove 70–80 % of metallothionein-bound Cu as Cu(I). The rest was resistant to reaction.

INTRODUCTION

Metallothionein (MT) is a sulphhydryl-rich, metal-binding protein. It is organized into two domains which individually bind multiple metal ions in metal–thiolate clusters [1]. MT is unique in that the nature of the bound metals and their stoichiometry of binding to the protein are dependent upon the history of the host cell [2–7].

The physicochemical properties of zinc- and cadmium-containing MTs have been studied since the protein was discovered more than 30 years ago [8,9]. There is general agreement about many of the chemical features of such MTs, beginning with the fact that 7 g-atoms of zinc, cadmium, or combinations of the two metal ions bind to the protein. In contrast, copper-bearing MTs have been examined less intensively and with less convergence of results [9]. For example, variable metal-to-protein stoichiometries have been reported. Cu-MT from Bedlington terriers with hereditary copper toxicosis was reported to contain 7–8 g-atoms of Cu per mol of protein, whereas protein from the liver of rats infected with copper bound 8–11 g-atoms of copper [10–12]. *In vitro* studies have shown that up to 12 Cu ions can react and bind to apo- or Zn-MT [13–16].

This report begins an examination of the properties of mammalian Cu-containing MT and focuses on the stoichiometry of binding of copper to MT isolated from calf liver. This source of protein was originally chosen because of its ready availability and the presence in the literature of a study on the distribution of Zn and Cu among the two clusters of calf liver MT [17]. It exists naturally as a mixed metal, Cu,Zn-protein with widely variable ratios of the two metals, so the question of apparent variable stoichiometry of metal binding can be addressed with native samples.

A key issue in the analysis of metal-to-protein stoichiometry in this protein is the fact that it is oxidatively unstable because of the co-existence of Cu and an abundance of sulphhydryl groups in the protein. Thus, methods to measure metal ions, total thiol and disulphide content, and copper ion oxidation state had to be applied to obtain reliable results.

MATERIALS AND METHODS

Materials

5,5'-Dithiobis-(2-nitrobenzoate) (DTNB) and 4,7-phenylsulphonyl-2,9-dimethyl-1,10-phenanthroline (BCS) were purchased from Sigma Chemical Co. Phenylmercuric acetate (PMA) was obtained from BDH Ltd. Other chemicals were reagent grade or of the highest purity available.

Purification of MT

Fresh bovine calf, veal calf, and fetal calf livers were purchased from a local slaughterhouse and stored at -80°C . For the preparation of MT, about 100 g of liver was thawed, cut into small pieces, and then rinsed in about 200 ml of 0.25 M sucrose, 6 mM 2-mercaptoethanol and 5 mM Tris/HCl buffer, pH 8.0. The liver was homogenized for 30–40 s at high speed and centrifuged at 48000 *g* for 60 min. The supernatant was loaded on a Sephadex G-75 column (12 cm $\times$ 85 cm) and eluted with 5 mM Tris/HCl/2 mM 2-mercaptoethanol, pH 8.0, at 4°C . All the solutions used in the course of isolation were degassed and bubbled with N_2 gas to keep the system in an anaerobic state. Further purification of MT was achieved by loading the MT band from Sephadex G-75 chromatography directly on to a

Abbreviations used: BCS, 4,7-phenylsulphonyl-2,9-dimethyl-1,10-phenanthroline; DTNB, 5,5'-dithiobis-(2-nitrobenzoate); MT, metallothionein (numerical subscripts refer to the number of metal ions bound; α and β signify the two domains of MT); PMA, phenylmercuric acetate; TNB, 5-thio-2-nitrobenzoate; EXAFS, extended X-ray absorption fine structure spectroscopy.

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21.5 mm × 15 cm DEAE 5PW semi-preparative ion-exchange column for HPLC separation using a gradient of 5 to 300 mM Tris/HCl, pH 7.4. Two separated components, MT 1 and MT 2, containing Cu and Zn, were either stored in the liquid state at 4 °C or were converted into the Cu,Cd-protein by reaction with stoichiometric amounts of CdCl₂. After displacement of Zn by Cd, metal ions not bound to the protein were removed with Chelex-100 and the Cd-MT was held at 4 °C as a liquid. No degradation in either Cu,Zn- or Cu,Cd-protein was observed over a 2 week period. For reasons not understood, Cu,Zn-MT lost variable amounts of Zn but not Cu when concentrated by ultrafiltration. This problem has been mentioned previously [18]. However, Cu,Cd-MT could be concentrated for NMR studies, described in the companion paper [19], using an Amicon YM2 membrane filtration system under an N₂ pressure of 138 kPa (20 lb/in²). Zn-MT was purified by similar procedures [20].

Amino acid analysis

MT 1 and MT 2 samples were desalted on a Econo-Pac 10DG desalting column of 10 ml bed volume (Bio-Rad Laboratories), using 10 mM NH₄HCO₃, pH 7.8. Protein fractions were then lyophilized. Amino acid compositions were determined at the Protein and Nucleic Acid Shared Research Facility of the Medical College of Wisconsin. The samples were treated first with performic acid for 2 h to oxidize cysteine to cysteic acid for later quantitative analysis. The protein was then subjected to hydrolysis in 6 M HCl at 110 °C for 20 h. The amino acid analyses were determined using a Beckman 6300 analyser.

Atomic absorption spectrophotometry

Metal analysis was carried out by flame atomic absorption spectrophotometry. Standard solutions of copper, cadmium and zinc were obtained from EM Industries, Inc.

Sulphydryl determination with DTNB

The sulphydryl content was determined using Ellman's reagent, DTNB. It reacts with the cysteine thiolate residues of the MT, releasing the 5-thio-2-nitrobenzoate (TNB) anion, which absorbs at 412 nm with an absorption coefficient of 13 600 M⁻¹·cm⁻¹ [20]. The reactions were conducted in 0.1 M phosphate buffer, pH 7.2, at 25 °C under anaerobic conditions. A vacuum system and stoppered sidearm cuvette were used to maintain anaerobic conditions.

Electrochemical detection of sulphydryl and disulphide groups

Amperometric titration of protein thiolates with PMA has been thoroughly described elsewhere [21]. Titrations were carried out anaerobically in 10 mM Tris/HCl and 0.1 M KCl at pH 8.6 in an electrochemical cell. The voltage of a dropping mercury electrode employed to monitor PMA concentrations was set at -0.5 V versus the saturated calomel electrode. After completion of reaction of PMA with sulphydryl groups in MT, 2.5 mM SO₃²⁻ was added to convert any available disulphide bonds into cysteinyl thiosulphonate and thiol groups. The latter reacted with PMA, revealing the concentration of disulphide in the sample.

Total oxidation state analysis with Fe(CN)₆³⁻

Ferricyanide is a moderately strong oxidant which has been used to titrate the total number of reducing equivalents in iron-sulphur

proteins, which contain Fe(II), thiol residues and inorganic sulphide [21]. In Cu,Zn-MT it is expected to react with Cu(I) and thiolate groups. Reactions were carried out anaerobically in 5 mM Tris/HCl, pH 7.2, in a stoppered sidearm cuvette. The change in absorbance of Fe(CN)₆³⁻ in the reaction mixture was measured at 420 nm. Using an absorption coefficient of 1000 M⁻¹·cm⁻¹, the number of reducing equivalents in the protein could be determined.

Reaction of Cu-containing MT with BCS

Ligand substitution reactions of BCS with Cu-containing MT were carried out in 0.1 M KCl/5 mM Tris/HCl at pH 7.4 under anaerobic conditions. The extent of formation of Cu(I)(BCS)₂⁻ was measured at 480 nm using an absorption coefficient of 13 500 M⁻¹·cm⁻¹.

RESULTS

Purification of Cu,Zn-MT 1 and 2

Sephadex G-75 chromatography of calf liver supernatant from a number of individual livers revealed a large band of metal in the 10 kDa region of the profile, consistent with the presence of MT (Figure 1). There was a surprising degree of variability in the Cu-to-Zn ratio, ranging from samples that contained mostly Cu-MT to primarily Zn-MT. This was even seen among samples derived from a single group of calves of similar age coming from the same farm. Repetitions of isolation of MT from one liver showed that MTs with reproducible metal ratios were obtained after G-75 and DEAE-Sephadex chromatography.

In Figure 1 no discrete bands of Zn or Cu bound to higher-molecular-mass protein were observed. This is because the amount of MT-bound Cu and Zn in these liver supernatants dwarfed other pools of these metals. In fact, the calculated numbers of mg of MT per gram wet weight of calf liver ranged from 1 to 4. Assuming about 200 mg of total protein per gram wet weight of liver, MT accounted for about 0.5 to 2 % of the protein. Hence, there is an enormous amount of MT in calf liver.

A typical fractionation of the MT peak with DEAE-Sephadex chromatography is shown in Figure 2. There were clearly species identifiable as MT 1 and MT 2 as well as a large, broad trailing band of Cu and Zn. The present study focused on MT 1 and MT

Figure 1 Sephadex G-75 chromatographic separation of calf liver supernatant

Elution performed with 5 mM Tris/HCl and 2 mM 2-mercaptoethanol at pH 8.0 and 4 °C. (■) Zn, (▲) Cu.

Figure 2 DEAE ion-exchange chromatographic separation of the 10000 Da band of Cu and Zn isolated by Sephadex G-75 chromatography

Chromatography was performed on samples as described in the Materials and methods section. (■) Zn, (▲) Cu.

Table 1 Amino acid composition of calf liver MT

	MT 1		MT 2	
Cys*	19.0	20†	18.7	20†
Asx	3.4	3	4.8	4
Thr	1.9	2	1.9	2
Ser	8.7	8	7.1	8
Glx	1.1	1	2.3	2
Pro	3.7	4	2.3	2
Gly	6.2	6	6.8	5
Ala	7.4	6	7.8	7
Val	1.9	2	1.8	2
Lys	5.7	7	5.6	8
Arg	1.0	1		
Met‡		1		1

* Cysteine determined as cysteic acid after performic acid oxidation.

† Reference [22].

‡ Met is destroyed by performic acid oxidation of these samples.

2 and did not examine the properties of the broad band of Cu and Zn which followed these discrete peaks.

The amino acid composition of the protein isolated from each of the two peaks of metal in Figure 2 was determined (Table 1). One can see that the amino acid contents of both isoforms experimentally measured in the present work are in excellent agreement with information in the literature on the compositions of bovine fetal calf liver MT 1 and MT 2 [22].

Sulphydryl and disulphide analysis of Cu,Zn-MT

Measurements of the sulphydryl content of Cu-containing MT 1 and MT 2 were used as a means to define accurately the protein concentration. Mammalian MT has 20 thiol groups per mol; so, with the sulphydryl analysis in hand, one can immediately calculate the concentration of MT protein. Initially, the reaction of MT with DTNB was used to quantify thiolate groups. Cu,Zn-MT reacts with slow, biphasic kinetics with this colorimetric reagent (Figure 3). Under the pseudo-first-order conditions of reaction (2 mM DTNB), the two rate constants for Zn₇-MT were 2.1×10^{-2} and $4.4 \times 10^{-3} \text{ s}^{-1}$. Those for Cu,Zn-MT were 1.3×10^{-2}

Figure 3 First-order plot of the kinetics of reaction of Cu,Zn-MT 2 (Cu/Zn = 0.75) with DTNB

MT (3 μM) was reacted with 2 mM DTNB in 0.1 M phosphate buffer, pH 7.2, and oxygen-depleted solutions. Faster rate process (□).

Table 2 Metal, sulphydryl, and reducing equivalents in Cu,Zn-MTs*

Isoform	Cu†	Zn†	SH‡		SH§/[Cu + Zn]	Reduction equivalents‡ (Fe(CN) ₆ ³⁻)	Reduction equivalents (SH + Cu)
			(DTNB)	(PMA)			
1	1920	1240	5180	4880	1.60		
			5170	4560	1.49		
			4850		1.54		
2	860	300		1950	1.68	2650	0.94
2	300	185		840	1.83	1310	1.10
				930	1.92		

* All concentrations are μM. In parentheses are the molecules reacting with Cu,Zn-MT in each column.

† Average of three measurements by atomic absorption spectrophotometry.

‡ Range of titration results among samples: ±10% for DTNB reaction; ±6% for PMA reaction, and ±5% for the Fe(CN)₆³⁻ reaction.

§ SH: average of results for DTNB and PMA, respectively.

and $1.3 \times 10^{-3} \text{ s}^{-1}$. Thus, the reaction was insensitive to changing the complement of bound metal ions as noted previously [20]. Table 2 lists the analytical data for representative samples of Cu,Zn-MT, including the thiol content per unit amount of Cu plus Zn as determined by the DTNB reaction for sulphydryl groups and atomic absorption spectrophotometry for the metal ions.

A potential problem in the use of this method for measurement of sulphydryl groups is that as they are titrated with DTNB, the Cu ions released from the protein might participate in the oxidation of TNB, the aromatic thiolate generated in the reaction of DTNB with SH residues. Indeed, when this reaction was carried out aerobically, the absorbance at 412 nm declined slowly after reaching its maximum value, consistent with the gradual oxidation of TNB. Because of the requirement of O₂ for this secondary process, DTNB reactions were carried out under anaerobic conditions.

Another limitation is that this method provides no information on the possibility that the protein contained some disulphide bonds which are indicative of copper-dependent oxidation of the native protein. Thus, a second method of sulphydryl quantification was used, mercurial titration in the absence and then

Figure 4 Titration of Cu,Zn-MT with PMA
MT (4 µM) was titrated with 6 mM PMA as described in the Materials and methods section. When Na₂SO₃ was added at the end of the titration, there was no drop in current.

Table 3 Metal composition and reactivity of Cu,Zn-MT

Isoform	Cu/Zn ratio	g-atoms of metal/mol of protein*	Percent fast reaction of Fe(CN) ₆ ³⁻	Percent Cu(I) chelation by BCS
2	20	15	76	
2	8.3	15		
2	5.0	15		87 (0.96)†
2	3.4	12		
2	2.9	12		88 (1.24)
2	2.2	11		
2	1.8	11	73	
1	1.6	11		
1	1.6	13		78 (1.76)
2	1.6	9.2		68 (1.12)
1	1.4	9.6		
2	0.67	6.9		
2	0.59	7.1	61	85 (0.40)
1	0.10	6.8	35	
2	0.07	6.9		
1	0.06	7.2		
2‡	0.00	6.6	7	

* Protein concentrations were calculated as [SH]/20.
† Number of Cu(I) ions unreacted with BCS in parentheses.
‡ Rabbit liver Zn₇-MT 2.

presence of sulphite, which also provided information on the concentration of disulphide in the sample. A titration of Cu,Zn-MT with PMA is shown in Figure 4. Typical of purified samples of Cu,Zn-MT 1 or -MT 2 less than 2 weeks old was the finding that there were almost no disulphide residues present. According to the results in Table 2, the correspondence between SH per unit content of Cu plus Zn in various samples of MT measured by the DTNB and PMA methods is good. The stoichiometry of Cu + Zn per mol of protein, based on the ratio of 20 mol of cysteine thiol per mol of MT, is listed for a number of samples of MT 1 and MT 2 in Table 3. It is evident that as the Cu/Zn ratio increased, the metal-to-protein ratio also increased from 7 to about 10–12 and finally to about 15. In the first category are samples with ratios of Cu to Zn of 0–0.67, suggesting that a significant fraction of bound metal can be Cu without changing the metal-to-protein stoichiometry of 7 found in Zn,Cd-MT. MT

Figure 5 First-order plots of the kinetics of reaction of rat liver Zn₇-MT 2 with ferricyanide
MT (4.3 µM) was reacted with 0.7 mM Fe(CN)₆³⁻ in 5 mM Tris/HCl at pH 7.2 under anaerobic conditions. Faster rate process (□). Eighteen mol of Fe(CN)₆³⁻ were consumed per mol of MT or per 20 SH groups.

with intermediate ratios of 10–12 contained Cu in higher amounts than Zn. At the upper end of 15 g-atoms of metal per mol of protein, almost all of the metal was Cu.

Oxidation of Cu,Zn-MT by ferricyanide

It has generally been thought that in native Cu-containing MT copper exists in the +1 oxidation state [23]. Thus, under anaerobic conditions it is likely that both SH and Cu(I) components of Cu,Zn-MT will react with Fe(CN)₆³⁻ to produce disulphides and Cu²⁺. If this is the case, the total number of oxidizable equivalents in Zn,Cu-MT will be equal to the sum of the SH and Cu contents of the protein as determined by other methods described above.

Figure 5 illustrates the kinetics of reaction of rat liver Zn₇-MT 2 with Fe(CN)₆³⁻. The zinc protein reacted almost entirely in two slow steps as measured by the loss of ferricyanide absorbance. In this reaction the number of equivalents of oxidant consumed per mol of thiol was 0.9, in excellent agreement with the expectation that each sulphhydryl group would undergo a one-electron oxidation by Fe(CN)₆³⁻.

The rate and stoichiometry of reaction of Cu,Zn-MT with Fe(CN)₆³⁻ were then examined. With protein containing Cu, some fraction of the total reaction occurred within the time of mixing. As seen in Table 3, this fraction grew when the ratio of Cu to Zn increased and approached 0.8–0.9 as copper became the predominant metal ion bound to the protein. However, under the conditions of reaction there was little if any effect of metal composition on the magnitude of the two slower, first-order rate constants, 1 × 10⁻² s⁻¹ and 1 × 10⁻⁵ s⁻¹. In addition, the endpoint of reaction corresponded well to the reaction of all of the protein thiolate ligands measured by the DTNB or PMA methods and plus the Cu determined by atomic absorption spectrophotometry. Thus, for eight different samples of MT, the ratio of (SH + Cu)/[Fe(CN)₆³⁻ reduced] = 0.96 ± 0.07. This redox titration experiment confirmed that all of the Cu in the native protein samples is in the +1 oxidation state. Further support that Cu, as well as thiols, had reacted came from the finding that native Cu,Zn-MT 1 had no detectable ESR signal at 77 K, but that the oxidized product has a signal with g_{||} = 2.246 and A_{||} = 180 G, typical of aqueous Cu²⁺ in the presence of nitrogen

Table 4 Metal-to-protein ratio in Cu-containing MT

Protein source	Cu/MT*	Cu/Zn†	Comments‡
Chicken liver	15	All Cu	Cu-substituted Cd,Zn-MT [29]
Rat liver	12(9)§ 5-6/ β -domain 5-6/ α -domain (4)§ 9, 9-11 8, 10 (4/ α)§ 6-12 5-6/ α 3-6/ β	All Cu All Cu All Cu 0.75	Reaction of Cu(I) with apo- or Zn-MT and apo- or Zn-domains [14-16] [11] [12] Reaction of Cu(I) with apo-domains, apo MT, and Cd ₅ , Zn ₂ -MT [30] [17,22] All Cu Cu-substituted Zn-MT 2 [31] Cu-substituted Zn-MT 2 [32] Titration of apoMT with Cu [33]
Rabbit liver	6, 12, and 12, 20 8, 12, 14	All Cu All Cu	
Rat kidney		0.75	[34]
Bedlington terrier liver	7-8	All Cu	[10]
Pig liver		0.8-2.5 2.0-2.5 4-7	[3] [25] [35]
Sheep liver		0.6-11	[18]
Fish liver		0.3-11	[26]
Ehrlich cells	7	All Cu	Cu-substituted Zn ₇ -MT [36]

* Homogeneous Cu-MT.

† Mixed-metal Cu,Zn-MT.

‡ Proteins isolated from tissues or cells unless noted.

§ Calculation based on metal displacement stoichiometry.

|| Summary of 11 species of fishes.

or nitrogen and oxygen ligands, probably from the Tris buffer and solvent water (results not shown) [24].

BCS reaction

A way to probe the reactivity of the Cu ions of Cu,Zn-MT was to react the protein with BCS, which forms a reddish-orange complex with Cu(I). Depending upon the sample of MT, the reaction proceeded with either mono- or bi-phasic kinetics. The kinetics were independent of BCS concentration and uniformly led to the removal of less than the total concentration of protein-bound Cu as measured by atomic absorption spectrophotometry (Tables 2 and 3). Since all of the copper appeared to be in the +1 oxidation state according to results of the reaction of ferricyanide with the protein, it seems that a minor fraction of the metal was not kinetically accessible to BCS. Among a number of samples in Table 3 about one Cu(I) ion in each sample (1.1 ± 0.5) did not react with BCS.

DISCUSSION

The intent of this work has been to define the stoichiometry of metal binding to naturally occurring mammalian MT when one of the metal ions is copper. The stimulus for this study came from the apparent differences in Cu-to-protein stoichiometry reported in a number of studies (Table 4). In particular, the results of Winge and co-workers, which focus on the titration of apoMT with Cu⁺, suggested that Cu⁺ binds to the protein with a different stoichiometry to Zn²⁺ or Cd²⁺, approaching 6 Cu(I) in each cluster [13-16]. It was hypothesized that Cu(I) and protein thiolates form clusters with trigonal metal-ligand co-ordination, in contrast with the tetrahedral co-ordination defined for zinc- or cadmium-containing MT [16]. Extended X-ray absorption fine structure spectroscopy (EXAFS) spectra of Cu,Zn-MT 2 (Cu/Zn

ratios of 5:2 and 6:3) from pig liver were best fitted with Cu bound to three sulphurs [25]. However, Briggs and Armitage [17] reported on a Zn₄Cu₃-MT from calf liver, in which copper was basically confined to the β -domain that ordinarily binds three Zn or Cd ions. This report argued that the β -domain was also occupied by three Cu ions. Indeed, Cu-MT from the livers of Cu-intoxicated Bedlington terriers was characterized as a Cu₇-s-protein and, according to EXAFS results, bound Cu with four sulphur ligands [10]. This study suggested that Cu may isomorphously replace Zn or Cd in MT. In addition, work with a variety of fish liver MTs containing Cu and Zn demonstrated that a wide range of Cu/Zn ratios could exist, which also brought into question the stoichiometry of Cu binding to MT [26].

In order to firmly establish the metal-to-protein stoichiometry, it was necessary to use procedures which provided precise analyses of both metal and protein. Standard flame atomic absorption spectrophotometry was employed to measure total Cu and Zn contents. Protein molarity was calculated by dividing the experimentally determined thiol concentration of MT by 20. The DTNB reaction with Cu,Zn-MT was routinely carried out under anaerobic conditions. This was because in the presence of O₂ the possibility existed that Cu liberated from the protein during the reaction would catalyse the oxidation of the TNB anion product that serves as a colorimetric indicator for SH groups. Indeed, slow oxidation of the TNB product was observed when this reaction was done in the presence of air.

A second, distinctly different method of thiol analysis was utilized, in which sulphhydryl groups were titrated with phenylmercuric ion. This technique also afforded the opportunity to assay the disulphide content of the protein. This was important because of the potential for oxidation in MTs containing redox-active Cu. It was shown that purified samples of MT 1 and MT 2 contained virtually no disulphides. This amperometric method gave values for SH concentration in very good agreement with the results from the DTNB reaction with MT. This provided assurance that the total sulphhydryl content of the protein was being assayed and that the protein concentrations derived from this measurement were accurate (Table 2).

Because of the importance of the metal and sulphhydryl analyses for this study, a different reaction was used to check the sum of the Cu(I) and SH content of MT samples. It had been shown previously that Fe(II), SH and inorganic sulphide of spinach ferredoxin react with Fe(CN)₆³⁻ stoichiometrically to produce Fe(III), cysteinyl sulphur groups at the oxidation state of disulphides, and sulphur in the zero oxidation state, respectively [27]. With Zn₇-MT, Fe(CN)₆³⁻ reacted slowly with biphasic kinetics reaching an endpoint of 2.70 equivalents per Zn or 19 per protein molecule, showing that virtually all of the SH groups are converted into disulphides. When Cu,Zn-MT was reacted with the oxidant, the stoichiometry was consistent with the reaction of all of the Cu plus the total complement of thiol residues in the protein. This experiment provided direct confirmation of the +1 oxidation state of all of the Cu in these proteins, a conclusion previously reached by others [23].

An interesting feature of the kinetics of the Fe(CN)₆³⁻ reaction with Cu,Zn-MT was that the fraction of reaction occurring in each step was a function of the Cu/Zn ratio in the sample. The fastest process, taking place within the time of mixing, increased with the Cu content of the protein, suggesting that facile oxidation-reduction of the SH groups of the protein proceeded through the intermediate reaction of Cu(I) with Fe(CN)₆³⁻ to generate an oxidatively unstable Cu(II)₂Zn-MT species. This route of reaction is not present in Zn₇-MT, thus explaining its overall slow kinetics of reaction.

The number of Cu(I) ions in various samples of Cu,Zn-MT which were reactive with a strong chelating agent for Cu(I), BCS, was also assessed. It was found in proteins ranging in Cu/Zn ratios from 0.02 to 5 that less Cu reacted with BCS than was present in the protein, according to atomic absorption spectrophotometric analysis (Table 3). In experiments not shown, the concentration of BCS was varied from 10 to 40 mM with no change in the amount of Cu(I)(BCS)²⁺ formed. This suggested that the incomplete reaction was not an equilibrium phenomenon. Instead, in each sample about one Cu(I) ion did not readily react with this competing ligand for Cu(I). Apparently, the protein is able to co-ordinate at least one Cu(I) in a manner that precludes its reaction with the competing BCS ligand.

The application of these analytical methods to a variety of samples of native Cu,Zn-MT has led to the body of information about the metal composition of these proteins shown in Table 3. Broadly, one sees that values for total metal per protein grouped themselves around 7, 10–12 and 15 g atoms per mol of MT, depending on the Cu/Zn ratio. At all ratios of metal to protein except 15, significant amounts of both Cu and Zn can be present. Apparently, at metal-to-protein ratios of 7, Cu may bind similarly to Zn. At larger ratios, other cluster geometries must be involved. Since this is the case, it is likely that many of the results summarized above in Table 4 are correct, though different from one another, and reflect differences in the physiological circumstances or chemical means leading to production of the proteins.

The distribution of Cu and Zn among the two domains of MT was not revealed by these studies. One does not know if these metals are primarily segregated into two homogeneous metal clusters or whether mixed metal clusters can exist. Another study in this series will address these questions [19].

Finally, it was noted in this work that Cu,Zn-MT was relatively stable even under aerobic conditions. The protein can be handled at room temperature for periods of several hours without noticeable degradation and can be stored anaerobically for at least 2 weeks at 4 °C without change. Copper ion is an excellent catalyst for the oxidation of small-molecule thiols by O₂ [28]. Considering that in MT large concentrations of Cu to SH are brought together, the metal cluster structure of the protein is surprisingly resistant to oxidation [9].

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