Articles

Products of Metal Exchange Reactions of Metallothionein†

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ABSTRACT: Hepatic metallothionein (MT) isolated from Cd-exposed animals always contains Zn (2–3 mol/mol of protein) in addition to Cd (4–5 mol/mol of protein), and the two metals are distributed in a nonuniform, but reproducible, manner among the seven binding sites of the protein's two metal–thiolate clusters. Different methodologies of preparing rabbit liver Cd,Zn-MT in vitro were investigated to provide insight into why such a distinct mixture of mixed-metal clusters is produced in vivo and by what mechanism they form. ^{113}Cd NMR spectra of the products of stepwise displacement of Zn^{2+} from Zn_7-MT by $^{113}Cd^{2+}$ show that Cd binding to the clusters is not cooperative (i.e., clusters containing exclusively Cd are not formed in preference to mixed-metal Cd,Zn clusters), there is no selective occupancy of one cluster before the other, and many clusters are produced with a nonnative metal distribution indicating that this pathway is probably not followed in vivo. In contrast, the surprising discovery was made that the native cluster compositions and their relative concentrations could be reproduced exactly by simply mixing together the appropriate amounts of Cd_7-MT and Zn_7-MT and allowing intermolecular metal exchange to occur. This heretofore unknown metal interchange reaction occurs readily, and the driving force appears to be the relative thermodynamic instability of three-metal clusters containing Cd. With this new insight into how Cd,Zn-MT is likely to be formed in vivo we are able for the first time to postulate rational explanations for previous observations regarding the response of hepatic Zn and metallothionein levels to Cd administration.

The low molecular weight, cysteine-rich metal-binding proteins called metallothioneins (MTs)[1] have attracted a great deal of interest in recent years because of their postulated importance in zinc and copper metabolism as well as in heavy-metal detoxification (Nordberg & Kojima, 1979). In mammals, MT biosynthesis is induced by several metal ions, notably Zn, Cd, and Cu, as well as by glucocorticoids, and the highest concentrations of protein are found in the liver and kidney (Squibb et al., 1977; Ohi et al., 1981; Bremner & Young, 1976; Karin & Herschman, 1980). Regulation of MT gene expression is now known to occur at the level of transcription (Durnam & Palmiter, 1981), although the molecular mechanism(s) of the induction process remains (remain) to be elucidated. A three-dimensional crystal structure of the protein has yet to be determined. However, on the basis of chemical and spectroscopic evidence considerable insight has been gained into the manner in which the protein interacts with its bound metal ions, especially Cd and Zn (Kägi & Vallee, 1961; Otvos & Armitage, 1980; Vasak & Kägi, 1983). All mammalian metallothioneins employ the thiolate sulfur atoms of the 20 cysteine residues in the 61 amino acid polypeptide chain as ligands for the 7 bound Cd or Zn ions. The positioning of the cysteine residues in strictly conserved locations in the sequence (Kägi et al., 1980) enables the thiolate ligands to adopt a unique configuration in which the metals are complexed in two separate polynuclear clusters (Otvos & Armitage, 1980, 1982). On the basis of an analysis of the ^{113}Cd–^{113}Cd scalar couplings that appear in ^{113}Cd NMR spectra of ^{113}Cd-labeled metallothioneins, the presence of a three-metal cluster requiring the participation of 9 cysteine sulfurs and a four-metal cluster requiring 11 cysteines has been demonstrated (Figure 1B). When both bridging and nonbridging cysteine ligands are used, the protein is able to provide each metal ion with a tetrathiolate coordination sphere with approximately tetrahedral symmetry (Otvos & Armitage, 1980; Vasak et al., 1981). Recent evidence suggests that the cysteine ligands of each cluster are contiguous in the amino acid sequence, leading to a two-domain model of the protein in which the amino-terminal half of the molecule (residues 1–30) forms the three-metal cluster and the carboxyl half forms the four-metal cluster (Winge & Miklossy, 1982; Boulanger et al., 1982).

Despite our improved understanding of metallothionein structure, we still know relatively little about the affinities of Cd and Zn for the multiple binding sites in the protein and the facility and mechanisms by which the bound metals may be transferred between sites and to other cellular ligands. Such fundamental information is needed to critically evaluate several postulates that have been made regarding the physiological function(s) of metallothionein. These include detoxification (via sequestration of toxic metals such as Cd and Hg), homeostasis of essential metals (primarily Zn and Cu), and active donation of essential metal ions to apometalloproteins (Piscator, 1964; Cousins, 1979; Udom & Brady, 1980; Li et al., 1980). Because of the two-cluster arrangement of metals bound to the protein and the observation that metallothionein in vivo usually contains two or more different metals distributed nonuniformly among the clusters (Otvos & Armitage, 1982; Briggs & Armitage, 1982), there is a strong possibility that metallothionein may perform multiple functions. For example, sequestration of toxic Cd might occur in one cluster

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[1] Abbreviations: MT(s), metallothionein(s); Cd_7-MT and Zn_7-MT, metallothionein in which all seven binding sites are occupied by Cd or Zn, respectively; Cd,Zn-MT, metallothionein whose seven binding sites are occupied by a mixture of Cd and Zn; MT-1 and MT-2, the two major isoprotein forms of metallothionein; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; DEAE, diethylaminoethyl.

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while Zn is stored in and perhaps actively donated to other cellular ligands from the other cluster. Such a hypothesis is compatible with the finding that animals subjected to brief, acute Cd administration do not produce Cd_7-MT but rather a Cd,Zn protein with a Cd/Zn ratio of approximately 2 that remains constant in the steady state of protein degradation and resynthesis for many months following Cd exposure (Winge et al., 1978). ^{113}Cd NMR analysis of the composition of this "native" Cd,Zn-MT from ^{113}Cd-injected rabbits indicates that the protein is not homogeneous but consists of a limited number of mixed-metal species (Otvos & Armitage, 1982). The Cd and Zn ions are not randomly distributed among the seven binding sites in these species but instead show a distinct preference for occupying the four- and three-metal clusters, respectively.

Before addressing the functionally significant question of why cells produce mixed-metal thioneins in response to Cd exposure, it is first necessary to understand *how* these species are produced in vivo. The mechanism of this process is not obvious in light of in vitro studies that show Cd^{2+} to have a substantially greater affinity than Zn^{2+} for *all seven* binding sites in the protein (Li et al., 1980; Vasak & Kägi, 1983). As a consequence, any free Cd^{2+} in vivo would be expected to outcompete Zn^{2+} for available binding sites in apothionein and quantitatively displace any Zn^{2+} bound to preexisting MT. The complete absence of any of the anticipated major product of these processes, Cd_7-MT, in isolated samples of Cd-induced metallothionein (Otvos & Armitage, 1982) argues against the importance of these events in vivo. Since it is thermodynamically untenable that Zn^{2+} could displace Cd^{2+} from selected sites of Cd_7-MT to produce the native mixed-metal clusters, we postulated that Cd induction might lead initially to the synthesis of Zn_7-Mt, followed by selective displacement of Zn from only the most reactive binding sites by substoichiometric amounts of available Cd^{2+}. To test this hypothesis, we have conducted a ^{113}Cd NMR analysis of the products of stepwise displacement of Zn from Zn_7-MT by $^{113}Cd^{2+}$, on the basis of which we conclude that native Cd,Zn-MT is unlikely to be produced by such a pathway. In the course of this study we have made the surprising discovery that Cd_7-MT *cannot* coexist in solution with Zn_7-MT without undergoing a facile intermolecular metal-exchange reaction that produces precisely the same mixed-metal species that are formed in vivo. These results strongly implicate Cd_7-MT and Zn_7-MT as obligatory intermediates in the formation of native Cd,Zn-MT. With this new insight into the reactivity of metallothionein in metal-exchange processes, a plausible explanation may now be presented for the mechanisms by which hepatic Zn and metallothionein levels in animals respond to Cd administration.

Materials and Methods

Protein Preparations. Native ^{113}Cd,Zn-MT-2 was isolated from the livers of ^{113}Cd-injected rabbits as previously described (Otvos & Armitage, 1980). Zn_7-MT-2 was purified from the livers of Zn-injected rabbits by the same protocol, except that separation of the MT-1 and MT-2 isoproteins on the DEAE-cellulose column was accomplished with a shallower 5–125 mM gradient of Tris-HCl, pH 8.6. Rabbits to which Zn was administered were injected once a day for 6 days with equal aliquots of 0.15 M $ZnSO_4$ to give a total dose of 0.32 mmol of Zn/kg of body weight. The animals were sacrificed 6 h after the final injection and the livers stored at −40 °C. $^{113}Cd_7$-MT-2 was prepared by direct displacement of the Zn^{2+} from purified Zn_7-MT-2 by stoichiometric amounts of added $^{113}Cd^{2+}$ (95 atom % from Prochem Isotopes). To prevent polymerization of the protein, which we have found is catalyzed by allowing concentrated solutions of metallothionein to coexist with free Cd^{2+} or Zn^{2+}, Chelex resin (Bio-Rad) was used to immediately remove the Zn^{2+} that is rapidly displaced (during the time of mixing) from the protein by $^{113}Cd^{2+}$. In a typical preparation of $^{113}Cd_7$-MT, 2 mL of a 5–10 mM solution of Zn_7-MT in 50 mM Tris-HCl, pH 7.5, is subjected to sequential additions of 1–2 protein molar equiv of $^{113}Cd^{2+}$ with the appropriate volume of a 0.5 M stock solution of $^{113}CdCl_2$. Immediately following addition of each aliquot of $^{113}Cd^{2+}$, the solution is mixed, 0.2 g of Chelex resin is added, and the Chelex is then rapidly removed by filtration. This entire procedure is performed in 5–10 s. Control experiments and the work of others (Vasak & Kägi, 1983) have demonstrated that under these conditions the Chelex removes 100% of the displaced Zn^{2+} without removing any of the protein-bound Cd^{2+} and Zn^{2+}. Cadmium and zinc concentrations in the protein were measured directly in water solutions by atomic absorption spectroscopy. Molar concentrations of pure MT samples were obtained spectrophotometrically by measuring their absorbance at 220 nm in 0.01 M HCl by using a molar absorptivity of the resultant apothionein of 47 300 (Bühler & Kägi, 1979).

NMR Methods. ^{113}Cd NMR spectra were recorded at 55.5 MHz on a Bruker WM-250 spectrometer using continuous broad-band proton decoupling. Metallothionein samples (3–7 mM) of ca. 1.8 mL were contained in 10-mm tubes in a 50 mM Tris-HCl, pH 8, buffer containing 10% D_2O to provide the field-frequency lock. Typical acquisition parameters were 70° pulse, 1-s pulse repetition rate, and 20 000–40 000 transients. Chemical shifts are reported in parts per million downfield from the ^{113}Cd resonance of 0.1 M $Cd(ClO_4)_2$. Spin–lattice relaxation times (T_1) were obtained by the inversion recovery method.

Results

Preparation and Spectrum of Homogeneous $^{113}Cd_7$-MT-2. The prevailing model of the metal–thiolate clusters in metallothionein, shown in Figure 1B, was based on an analysis of the ^{113}Cd–^{113}Cd spin-coupling connectivities in spectra of native rabbit liver ^{113}Cd,Zn-MT and $^{113}Cd_7$-MT produced in vitro from the native protein (Otvos & Armitage, 1980, 1982). Since both of these protein preparations were heterogeneous, the former because of Zn occupation of several binding sites in some, but not all, of the protein molecules (Otvos & Armitage, 1982) and the latter because of partial metal ion deficiency (Otvos & Armitage, 1980) and/or heterogeneity in amino acid sequence (Armitage et al., 1982), their ^{113}Cd NMR spectra contained more than the 7 resonances expected on the basis of the number of metal binding sites (for example, the spectrum of ^{113}Cd,Zn-MT-2 shown in Figure 2A contains 15 resonances). Because of the spectral complexity of these heterogeneous systems, some investigators have questioned the validity of the two-cluster model derived from analyses of such ^{113}Cd NMR data (Vasak et al., 1985).

In Figure 2B–D are presented spectra of $^{113}Cd_7$-MT-2 prepared by a new procedure that prove that metallothionein can be obtained in a verifiably homogeneous state and that provide unambiguous support for the two-cluster model in Figure 1B. The seven resonances that appear in these spectra are well separated from one another above room temperature, have virtually identical integrated areas, and contain well-resolved multiplet fine structure that is only compatible with the ^{113}Cd–^{113}Cd spin-coupling interactions depicted in the cluster representations in Figure 1. In particular, it can be noted that the complex, but reproducible, multiplet structures of resonances VI and VII can only be accurately simulated

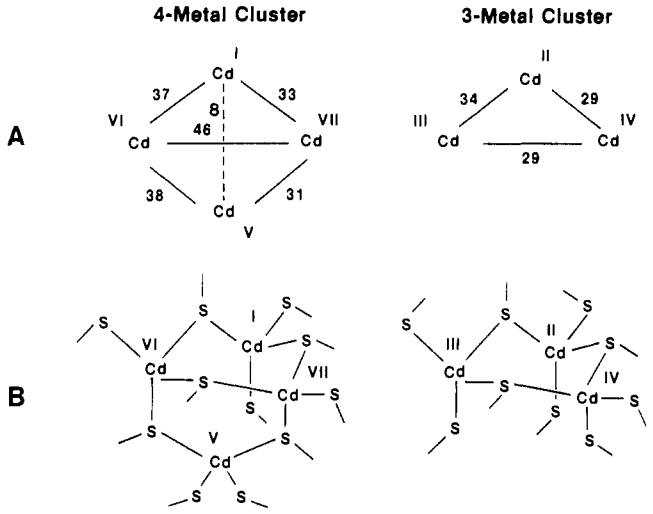

FIGURE 1: (A) Schematic representations of the metal clusters in Cd_7-MT showing the ^{113}Cd–^{113}Cd two-bond (solid lines) and four-bond (dashed line) scalar coupling interactions between metals. The Roman numeral beside each Cd refers to the corresponding resonance in the ^{113}Cd spectrum in Figure 2D, and the numbers on each line are the observed ^{113}Cd–^{113}Cd coupling constants. (B) Postulated structures for the metal clusters in Cd_7-MT based on ^{113}Cd chemical shift and spin coupling data (Otvos & Armitage, 1980).

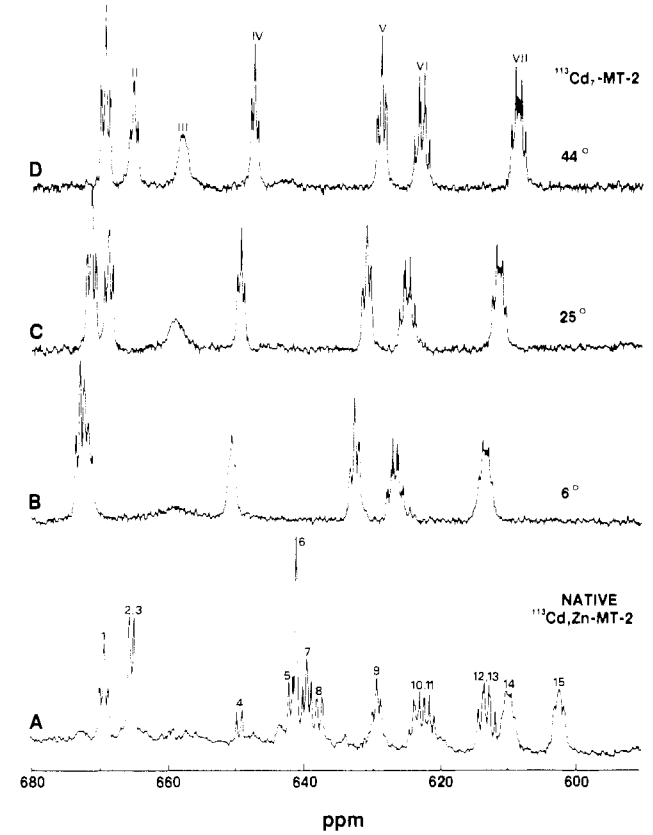

FIGURE 2: ^{113}Cd NMR spectra of (A) native ^{113}Cd,Zn-MT-2 (Cd/Zn ratio = 1.9) at 24 °C and (B–D) $^{113}Cd_7$-MT-2 prepared by in vitro displacement of Zn^{2+} from Zn_7-MT-2 by $^{113}Cd^{2+}$ at the temperatures indicated.

by employing the inequivalent two-bond coupling interactions that each metal has with the other three occupants of the four-metal cluster shown in Figure 1A.

The temperature dependences of the chemical shifts of the seven resonances in Figure 2B–D and their spin–lattice relaxation times (T_1) are compiled in Table I. Considerable differences in these parameters are noted among the resonances, reflecting the uniqueness of coordination environment

Table I: Chemical Shifts and Spin–Lattice Relaxation Times of ^{113}Cd Resonances of Cd Metallothionein 2

resonance[a]	chemical shift (ppm) 6 °C	25 °C	Δ_{25-6} °C	44 °C	Δ_{44-6} °C	T_1 (s)[b]
I	673.0	671.3	−1.7	669.4	−3.6	0.68
II	671.8	668.8	−3.0	665.1	−6.7	0.53
III	658.7	659.0	+0.3	657.9	−0.8	0.75
IV	650.5	649.2	−1.3	647.2	−3.3	0.75
V	632.6	630.9	−1.7	628.8	−3.8	1.24
VI	626.8	625.0	−1.8	622.8	−4.0	1.27
VII	613.3	611.3	−2.0	608.4	−4.9	0.22

[a] The resonance numbering scheme corresponds to that used in Figure 1. [b] T_1 values were determined at 30 °C with the same sample used to provide the spectra in Figure 1B–D.

at each metal binding site. The observed differences in T_1 are difficult to ascribe to specific structural features of the sites without a much more extensive field-dependent study to separate the relative contributions to relaxation from dipolar and chemical shift anisotropy mechanisms (Armitage & Otvos, 1982). However, from a practical standpoint it is worth noting that since all T_1 values are quite short, spectra can be accumulated relatively efficiently. The chemical shifts of all seven resonances are temperature-dependent, with those of the four-metal cluster metals (I and V–VII) all shifting upfield by ca. 0.1 ppm/°C. From the magnitude of this temperature dependence, which is similar to that reported for several organocadmium compounds (Kennedy & McFarlane, 1977), and the fact that the line widths remain virtually unaffected, we believe that the origin of the effect is the "inherent" temperature dependence on nuclear shielding experienced by all heavy nuclei that have large paramagnetic components to their shielding tensors (Harris & Mann, 1978). In contrast, the resonances of the three-metal cluster (II–IV) clearly experience a temperature-dependent chemical-exchange contribution to both their shifts and their line widths. Most noteworthy are resonance II, which shifts upfield almost twice as far as the other resonances between 6 °C (where it overlaps with resonance I) and 44 °C, and resonance III, whose line width at 6 °C is almost broadened beyond detection but at higher temperatures is much narrower. This transition from intermediate- to fast-exchange regimes between 6 and 44 °C is independent of pH (between 6 and 10), ionic strength (to 0.5 M NaCl), and protein concentration (1–15 mM) and is therefore attributed to an as yet undefined intramolecular fluctuation of the environment of the three-metal cluster domain of the protein. It should be noted that, throughout the temperature range, the spin-coupling interactions made by resonance III with resonances II and IV remain resolved. This puts a lower limit of ca. 0.01 s on the lifetimes of the metals in their respective sites of the three-metal cluster and precludes rapid dissociation and reassociation of metal from the cluster as being the origin of the line width and shift modulations of their resonances.

Titration of Zn_7-MT with $^{113}Cd^{2+}$. Several lines of evidence suggest that the two clusters of metallothionein exhibit differential affinities for Cd, with the tighter binding occurring at the four-metal cluster (Otvos & Armitage, 1980; Nielson & Winge, 1983; Armitage & Boulanger, 1983; Vasak & Kägi, 1983). Assuming there are no kinetic barriers to the formation of the thermodynamically favored species, it would therefore be anticipated that in the early stages of a stepwise Cd^{2+} displacement of Zn^{2+} from Zn_7-MT the preponderance of Cd would be bound to the four-metal cluster. Furthermore, if Cd_4 clusters are appreciably more stable than clusters containing a mixture of Cd and Zn, a cooperative displacement of Zn

from the four-metal clusters would be expected. These predictions are easily tested by monitoring by ^{113}Cd NMR the mixed-metal products of a $^{113}Cd^{2+}$ titration of Zn_7-MT. The utility of such an approach was demonstrated in a previous study of Cd-induced native Cd,Zn-MT-2 in which the chemical shifts and spin-coupling connectivities of the ^{113}Cd signals were used to assess how many discrete cluster species were present and what their metal compositions were, while the integrated areas of the resonances provided a measure of their relative concentrations (Otvos & Armitage, 1982). Figure 2A shows a typical ^{113}Cd spectrum of native ^{113}Cd,Zn-MT-2 and in Figure 5 are depicted the six Cd-containing cluster species proposed to be present in the heterogeneous mixed-metal protein. Three four-metal cluster species (A, C, and D) account for the preponderance of bound Cd (81%) and give rise to 10 of the 15 resonances. Most of the remainder of the Cd is found in a three-metal cluster (F) in which the other two sites are occupied by Zn (Otvos & Armitage, 1982). A significant observation has been that, despite wide variations in the protocols used to prepare the ^{113}Cd-induced protein (variation of dose and duration of ^{113}Cd administration, variation from 1 day to 2 weeks of the time between final ^{113}Cd injection and sacrifice of the animal, and variation of the purification procedure), ^{113}Cd spectra of a given isoprotein of native MT are always identical. This strongly supports the assumption that the metal distribution of the isolated protein as determined by ^{113}Cd NMR is a true reflection of the steady-state metal distribution in vivo rather than an artifact caused, for example, by metal exchange during isolation of the protein. The spectrum of native ^{113}Cd,Zn-MT-2 has therefore been employed by us as a "spectral fingerprint" of the characteristic in vivo distribution of Cd and Zn among the cluster sites to determine whether the same distribution (and hence spectrum) can be generated in vitro via $^{113}Cd^{2+}$ titration of Zn_7-MT-2. If so, it would constitute strong evidence that either (1) the metal compositions and relative concentrations of the mixed-metal cluster species generated in vivo and via Zn^{2+} displacement by Cd^{2+} in vitro are both controlled solely by the relative thermodynamic stabilities of the cluster species or (2) the mixed-metal clusters are formed in vivo and in vitro by the same kinetically controlled pathway.

In Figure 3 are shown the results of a $^{113}Cd^{2+}$ titration of Zn_7-MT-2. Although the spectra generated are quite complex and not fully interpretable without ^{113}Cd decoupling data to establish all of the spin-coupling connectivities, there are several important conclusions that can be immediately drawn. First, it is apparent that in the early stages of the titration (Figure 3A–C) neither the four- nor the three-metal cluster is occupied selectively or cooperatively by Cd. In fact, just the opposite seems to be the case. Since numerous resonances in these spectra appear at positions where no resonances have previously been observed in native protein samples and most of these contain relatively few ^{113}Cd–^{113}Cd spin–spin splittings, it can be concluded that mixed-metal clusters are formed in preference to all-Cd clusters. Only late in the titration (Figure 3D–F) do the resonances attributable to ^{113}Cd in all-Cd three-metal clusters (II–IV) and four-metal clusters (I and V–VII) appear, and both sets of signals gain intensity approximately in tandem as the titration progresses. Second, it is concluded that very early in the titration there is considerable site selectivity of Zn displacement. In Figure 3A only two ^{113}Cd resonances appear and both are singlets, signifying that they arise from clusters containing a single ^{113}Cd ion flanked by Zn neighbors. The chemical shift of the largest of these two resonances is identical with that of the only singlet

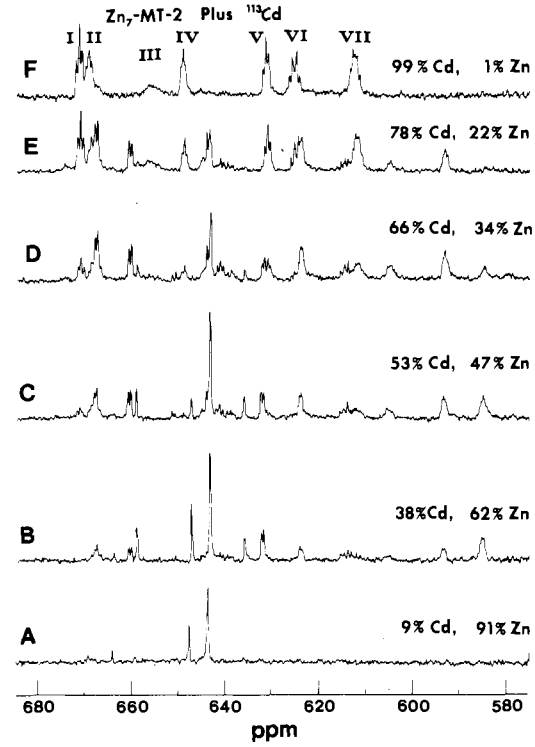

FIGURE 3: ^{113}Cd NMR spectra at 15 °C of ^{113}Cd,Zn-MT-2 of variable Cd and Zn composition produced by titration of Zn_7-MT-2 with $^{113}Cd^{2+}$. After addition of each successive aliquot of $^{113}Cd^{2+}$, followed by immediate Chelex resin treatment as described under Materials and Methods, spectra A–F were acquired. Atomic absorption analysis of the protein at each stage of the titration, giving the metal compositions to the right of each spectrum, was consistent with the stoichiometric displacement of Zn^{2+} by each aliquot of added $^{113}Cd^{2+}$.

in the native ^{113}Cd,Zn-MT-2 spectrum (resonance VI in Figure 2A) and for this reason is believed to originate from ^{113}Cd in the three-metal cluster designated F in Figure 5.

It is of fundamental interest to understand why so very few Cd-containing cluster species are formed after addition of a small amount of Cd^{2+} to Zn_7-MT, since there are potentially many more different species that can result from a purely random distribution of Cd among the seven binding sites. The two limiting possibilities are (1) that the system during the course of the titration is in a thermodynamic equilibrium state in which only a few mixed-metal clusters have appreciably greater stability than the others and (2) that the observed cluster composition reflects a nonequilibrium distribution that is kinetically controlled by differences in the ease of Zn^{2+} displacement by Cd^{2+} from certain sites. If it were possible to "tag" the Cd^{2+} added in the first step of the titration to distinguish it from the Cd^{2+} added subsequently, one could distinguish between the two possibilities by determining whether the tagged Cd^{2+} remained bound to its initial binding sites when additional Cd^{2+} was added (signifying kinetic control) or whether complete scrambling of "tagged" and "untagged" Cd among the sites occurred (thermodynamic control). Fortunately, such a "tagging" experiment can be performed with Cd NMR by employing different NMR-detectable isotopes, ^{113}Cd and ^{111}Cd, in successive steps of the titration. Several such experiments were conducted (results not shown), and in each case the results were the same: both the ^{111}Cd and ^{113}Cd NMR spectra of the resulting ^{111}Cd, ^{113}Cd,Zn-MTs were identical, indicating that complete equilibration of the Cd isotopes among clusters of different thermodynamic stability had occurred.

The final, and perhaps most significant, conclusion that may be drawn from the data in Figure 3 is that at no point in the

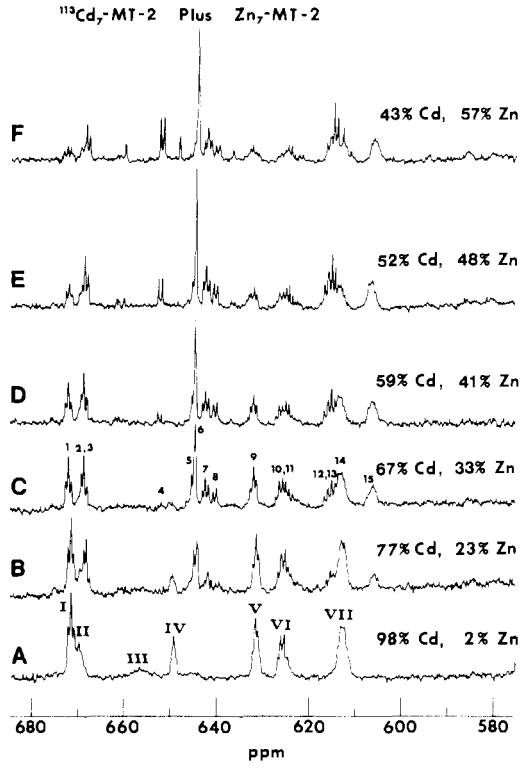

FIGURE 4: ^{113}Cd NMR spectra (30000 transients each) at 12 °C of ^{113}Cd,Zn-MT-2 of variable Cd and Zn composition produced by titration of $^{113}Cd_7$-MT-2 with Zn_7-MT-2. Spectrum A is of 3 mM $^{113}Cd_7$-MT-2, which still contains a trace (2%) of Zn after its preparation from Zn_7-MT-2 as described under Materials and Methods. Spectra B–F result from successive additions of lyophilized Zn_7-MT-2 to this protein, giving the metal compositions to the right of each spectrum.

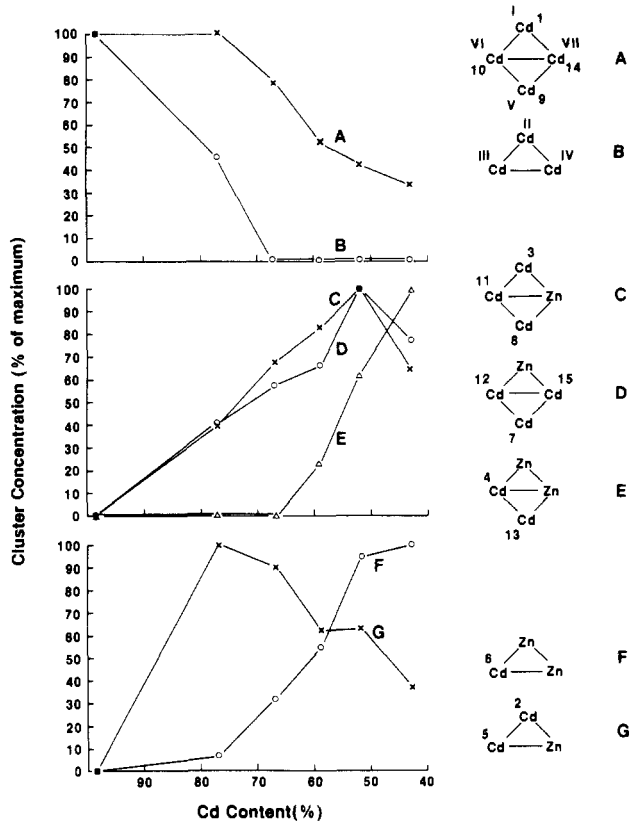

FIGURE 5: Changes in relative concentrations of metal cluster species during the titration (Figure 4) of $^{113}Cd_7$-MT-2 with Zn_7-MT-2. The schematic structures on the right represent the six Cd-containing cluster species (A and C–G) that have previously been postulated to be formed in vivo in response to Cd administration on the basis of an analysis of the ^{113}Cd,Zn-MT-2 spectrum (Otvos & Armitage, 1982) and that are also formed in the titration experiment in Figure 4. The number beside each Cd in these structures corresponds to the ^{113}Cd resonance it produces in the spectra of $^{113}Cd_7$-MT-2 (Figure 2D or 4A) and ^{113}Cd,Zn-MT-2 (Figure 2A or 4C). The relative concentrations of the cluster species plotted on the left were derived from the integrated areas of these resonances during the course of the titration.

titration is the characteristic spectral "fingerprint" of native ^{113}Cn,Zn-MT-2 generated. At the Cd/Zn ratio of ca. 2 that is exhibited by virtually all Cd-induced MTs, the spectrum of the protein prepared in vitro by ^{113}Cd titration (Figure 3D) bears little resemblance to that of the native protein produced in vivo (Figure 2A). The spectrum in Figure 3D, for example, contains prominent signals at 594 and 585 ppm, which are at lower frequency than any resonances previously reported for any native rabbit liver MT preparation. On the basis of this evidence we do not believe that the in vivo pathway to the characteristic mixed-metal cluster distribution found in native Cd,Zn-MT is via Cd^{2+} displacement of Zn^{2+} from Zn-MT.

Titration of $^{113}Cd_7$-MT with Zn_7-MT. The detailed mechanism by which Zn^{2+} is so readily displaced from metallothionein by Cd^{2+} to generate thermodynamically favored mixed-metal cluster species has not yet been completely established but involves the active participation of the free Cd^{2+} in assisting dechelation of the Zn^{2+} (R. West and J. D. Otvos, unpublished observations). We therefore doubted that, without the intermediacy of free metal ion, a facile kinetic pathway would exist for the interchange of metals between Zn_7-MT and Cd_7-MT, even though we suspected that such a process might thermodynamically favor the production of more stable mixed-metal clusters. As shown by the data in Figure 4, our doubts were unfounded: metal is indeed exchanged very readily[2] between metallothionein molecules in the absence of free metal. More importantly, not just any stable mixed-metal clusters are produced by this process, but *only* those having precisely the same metal compositions and relative concentrations as those produced in vivo in response to Cd administration. This conclusion is reached by comparing the spectrum of the ^{113}Cd,Zn protein (Cd/Zn = 2) in Figure 4C with that of the native ^{113}Cd,Zn protein in Figure 2A. Clearly, these two "fingerprints" are virtually identical, and both differ markedly from the spectrum of the ^{113}Cd,Zn protein with the same Cd/Zn ratio in Figure 3D. The ability to so accurately reproduce in vitro via direct metal interchange between metallothioneins the complex and distinctive distribution of mixed-metal cluster species produced in vivo lends very strong support to the notion that the two processes share a common mechanism.

Insight into the driving force for the interprotein metal exchange reaction is provided by the plots in Figure 5 of the relative concentrations of the cluster components of the ^{113}Cd,Zn-MT produced during the course of the titration. Although detailed interpretation of these data is hindered by an inability to distinguish between clusters located in the Cd-MT "donor" molecules and the Zn-MT "acceptors", it can be concluded that in the early stages of the titration Cd is transferred mainly from three-metal clusters of Cd-MT to four-metal clusters of the Zn protein. For example, at the stage of the titration where the Cd/Zn ratio has reached 2 (Figure 4C), the concentration of the all-Cd three-metal cluster (species B) has decreased to zero in parallel with a large rise in concentration of the Cd,Zn four-metal cluster species C and D. At the same point in the titration only a small decrease

[2] Preliminary data on the rate of metal exchange between Cd_7-MT-1 and Zn_7-MT-2 indicate that the time scale of the reaction is minutes or less.

(20%) has occurred in the concentration of the all-Cd four-metal cluster, species A. These and similar data obtained with MT-1 suggest that the surrounding protein structure imposes differential steric constraints on the two clusters that in general favor binding of the smaller Zn^{2+} ion to the three-metal cluster and the larger Cd^{2+} ion to the four-metal cluster. What is noteworthy about the results in Figures 3 and 4 is not that the metal composition of the clusters influences their stability but rather that a facile mechanism of interprotein metal exchange apparently exists to permit mixed-metal clusters of greater stability to be produced from clusters of relatively less stability. The biological implications of this unexpected new reaction of metallothionein are discussed below.

Discussion

It is well established that Cd^{2+} binds about 10000 times as tightly to metallothionein as Zn^{2+} (Vasak & Kägi, 1983) and, as a consequence, Zn^{2+} bound to metallothionein can be quantitatively displaced by addition of a stoichiometric amount of Cd^{2+} to produce an all-Cd protein. When $^{113}Cd_7$-MT from a variety of sources has been prepared in this manner and analyzed by ^{113}Cd NMR, the protein has always been found to be inhomogeneous as judged by the presence of more than seven resonances in the spectra and the observation of subintegral intensities for the three-metal cluster signals (Otvos & Armitage, 1980; Armitage et al., 1982; Vasak et al., 1985). The spectra presented here of rabbit liver Cd_7-MT-2 are, in contrast, clearly indicative of a genuinely homogeneous protein in which the metals in the two Cd–thiolate clusters are arranged as depicted in Figure 1. The structures of these clusters can now be unambiguously confirmed by the distinctive ^{113}Cd–^{113}Cd splitting patterns of the resonances (which were always partially obscured by signal overlap in previous spectra of inhomogeneous protein samples) and by two-dimensional ^{113}Cd correlated (COSY) spectra (not shown), which verify the spin-coupling connectivities previously established by selective decoupling experiments (Otvos & Armitage, 1980).

The key to preparing homogeneous Cd_7-MT by in vitro Zn^{2+} displacement from native Zn_7-MT was our discovery that the protein undergoes a time-dependent oligomerization in the presence of free metal ion. By rapid removal of excess Cd^{2+} or Zn^{2+} from the protein solution by treatment with Chelex resin, the production of dimers and higher molecular weight species can be largely avoided. Oligomers of ^{113}Cd-MT manifest themselves in ^{113}Cd NMR spectra by exhibiting three-metal cluster resonances whose intensities are greatly attenuated or sometimes completely absent (presumably due to severe line broadening from unknown chemical-exchange processes) and four-metal cluster signals with chemical shifts different from those of the monomeric protein (H. R. Engeseth and J. D. Otvos, unpublished observation). Although studies to determine the structures and mechanism of formation of the oligomers are still in progress, we have found that treatment with dithiothreitol is capable of converting MT dimers into monomers, though it is less effective in reversing the oligomerization of larger species.

The prevention of oligomer formation during in vitro production of Cd_7-MT was of practical importance to us in the studies reported here but was not our primary objective. Rather, we were interested in shedding some light on the interrelationship between metallothionein's putative involvement in Zn metabolism and in protecting against the toxic effects of Cd. By virtue of coordinating its bound metal ions in two distinct clusters, each possessing a differential affinity for Cd and Zn (Otvos & Armitage, 1982; Briggs & Armitage, 1982), metallothionein offers at least the potential for performing both of these physiological functions. If so, it is possible that Cd and perhaps other heavy metals at least partially exert their toxicity by displacing Zn from essential binding sites in metallothionein, thereby interfering with normal Zn metabolism.

Two significant observations relevant to this hypothesis have been made in studies of the physiological responses of rats to Cd exposure (Winge et al., 1975, 1978) and in subsequent studies of other animals. First, Cd administration was found to lead not only to increased Cd levels in the liver but also to increased Zn levels that were proportional to Cd dose (Winge et al., 1975). This tissue "mobilization" of Zn follows that of Cd by several hours, and both the Zn and Cd are bound primarily to metallothionein (Winge et al., 1978). Second, it was found that the metal composition of Cd-induced MT varies with time following induction. In the first hours after acute Cd administration the isolated rat liver protein contains predominantly Cd and little or no Zn. Over a period of 12 h, however, the Zn content of MT rises substantially, concomitant with Zn mobilization into the liver, to ultimately give protein with a Cd/Zn ratio of approximately 2. This ratio is then maintained through many cycles of protein degradation and resynthesis for at least 6 months (Winge et al., 1978). Although no satisfactory explanations for these events were apparent at the time, the authors suggested that they were manifestations of metallothionein's primary physiological role in Zn metabolism.

The only information currently available regarding the manner in which Zn and Cd are partitioned in vivo among the seven binding sites of a Cd-induced metallothionein comes from ^{113}Cd NMR studies of native rabbit liver MT having the characteristic 2:1 Cd to Zn ratio (Otvos & Armitage, 1980, 1982). As described in these studies, spectra such as in Figure 2A provide a sensitive gauge not only of Cd occupancy of each site but also, via analysis of spin-coupling patterns, of the identities and locations of the metals that share occupancy of each metal–thiolate cluster. The results of these studies indicate that native Cd,Zn-MT is a heterogeneous mixture of protein species containing a very limited, but characteristic, number of different mixed-metal clusters, the postulated compositions of which are given in Figure 5. The relative concentrations of the mixed-metal clusters are given by the relative intensities of the ^{113}Cd signals, which are observed to be invariant in protein induced by either short- or long-term Cd exposure. How and why such a distinctive distribution of metals is formed in vivo and then maintained for long periods of time are not clear, particularly in light of thermodynamic binding studies which indicate that the protein has a much greater affinity for Cd^{2+} than for Zn^{2+}. This fact would suggest that the major species of new protein synthesized in response to Cd induction would be Cd_7-MT, yet spectra of native Cd,Zn-MT show that *none* of this species is present (Otvos & Armitage, 1982). To resolve this apparent paradox and hopefully provide insight into the intracellular events that govern the steady-state distribution of Cd and Zn among the clusters in MT, we set out to find in vitro conditions that would produce protein with the same unique distribution of mixed-metal clusters that is formed in vivo, using as a gauge of success the ability to reproduce the ^{113}Cd spectral "fingerprint" of native ^{113}Cd,Zn-MT.

The very significant and unexpected result of this endeavor has been the discovery that metallothionein participates in a new type of reaction, a direct metal interchange reaction that produces mixed-metal clusters apparently without the intermediacy of free metal ion. Simply by mixing together the

appropriate amounts of Cd_7-MT and Zn_7-MT and allowing intermolecular metal redistribution to occur, we could reproduce the exact metal distribution of the native Cd-induced protein (Figure 4). Adjusting the relative amounts of the Zn- and Cd-containing proteins to give Cd/Zn ratios higher or lower than 2 produces protein species that clearly exhibit "nonnative" ^{113}Cd spectra. Similarly, all attempts to reproduce the native metal distribution by site-selective displacement of Zn^{2+} from metallothionein by Cd^{2+} also failed (Figure 3), implying that this is not the in vivo route to formation of mixed-metal clusters. If, as now appears likely, the actual route is via the protein–protein metal-exchange reaction, the data strongly indicate that Cd-induced metallothioneins exhibit steady-state Cd/Zn ratios of 2 not because of any special stability associated with species containing this particular metal composition but rather because, under as yet unknown influences, the liver synthesizes just the right amount of metallothionein to maintain these levels of protein-bound Cd and Zn.

The reason metal interchange takes place between the clusters of metallothionein appears to be the increased stability that results from formation of certain mixed-metal clusters from mixtures of clusters containing solely Cd or Zn. The data in Figure 5 suggest that the main driving force for the reaction is the relative thermodynamic instability of the all-Cd three-metal cluster, which accounts in large part for the observed preponderance of Cd located in four-metal clusters in native Cd,Zn-MT. Since we are accustomed to thinking of Cd-MT as a much more stable species than Zn-MT on the basis of thermodynamic binding studies (Vasak & Kägi, 1983), it might seem surprising at first that metal exchange between the two species occurs at all. However, it must be recognized that two completely different reactions are being compared. In the binding reactions, what is being measured are the relative affinities of the binding sites in MT for Zn^{2+} vs. Cd^{2+}. Cd^{2+} binds tighter than Zn^{2+} owing primarily to the relatively greater strength of Cd–thiolate bonds compared to Zn–thiolate bonds. In the metal-exchange reactions, all of the metals are bound to begin with and no net change occurs in the number of Cd– and Zn–thiolate bonds. The question is simply whether the energy of the system can be lowered by a redistribution of Cd and Zn among specific sites, and the answer is apparently yes. It is very important to note, however, that thermodynamics is not the sole determinant of which mixed-metal clusters are formed and in what amounts in metallothionein samples with a particular Cd and Zn composition. The existence of an accessible kinetic pathway between the exchanging species is also required. A comparison of the very different spectra in Figures 3D and 4C illustrates this point. Despite having identical Cd/Zn ratios of 2, the protein samples producing these spectra clearly contain mixed-metal clusters having both different metal compositions and relative concentrations. If pathways were accessible for interprotein metal interchange between all sites in the two clusters, the same thermodynamic equilibrium distribution of cluster species would have resulted regardless of the method of protein preparation and identical spectra would have been obtained. Our finding that the ultimate distribution of Cd and Zn among the multiple binding sites in metallothionein appears to be strongly dependent on kinetic factors (mechanism of exchange) as well as differences in cluster stability lends especially strong support for the proposition that direct metal interchange between Cd_7-MT and Zn_7-MT is in fact the same pathway by which native Cd,Zn-MT is formed in vivo.

The knowledge that Zn_7-MT cannot coexist with Cd_7-MT without undergoing metal interchange to produce four- and three-metal clusters occupied preferentially by Cd and Zn, respectively, helps explain many previous observations regarding hepatic responses to Cd administration and offers new insights into the interrelationship between the postulated dual roles of metallothionein in Zn metabolism and Cd detoxification. As a stimulus to future research in this area, we offer the following scenario for the participation of MT in the molecular events that take place in the liver in response to a single acute dose of Cd. First, in accord with recent experiments (Day et al., 1984) and the thermodynamic preference of metallothionein for Cd, whatever basal Zn-MT is present is rapidly converted to Cd-MT via Zn displacement from the protein by Cd entering the liver. At this time, possibly triggered by the absence of Zn-MT or the presence of excess unsequestered Cd, apothionein synthesis is induced. The protein is synthesized at an elevated rate until all of the hepatic Cd has been sequestered as Cd-MT. Despite having now "detoxified" the Cd, the liver continues to produce apothionein in amounts far exceeding those needed to reestablish the original basal level of Zn-MT. Since insufficient intracellular metal is available to coordinate the excess thionein, Zn for this purpose is drawn into the liver from the plasma, or "mobilized" (Winge et al., 1978). Continuation of this process is responsible for the progressive decrease observed in the Cd/Zn ratio of isolated MT during the first 12 h after Cd administration (Winge et al., 1978). The subsequent leveling off of the Cd/Zn ratio when a value of approximately 2 is reached is the result of a decrease in the rate of thionein synthesis at this point. Thionein is synthesized from this time on only at a rate necessary to replenish protein lost to normal biodegradation, which effectively maintains indefinitely the elevated concentrations of MT, Cd, *and* Zn in the liver.

To adequately explain why hepatic thionein production "overshoots" the level required to sequester the administered Cd and then is required to maintain a steady-state metallothionein Zn concentration roughly half that of Cd would clearly require a much fuller understanding of the mechanism of MT induction than currently exists. We suggest, however, that underlying these events is metallothionein's essential role in Zn metabolism coupled with the inability of the protein to perform that function when Zn-MT is in the presence of much larger concentrations of Cd-MT. As we have shown, this situation results in metal exchange leading to the production of mixed-metal clusters. If it is postulated that the metabolic function(s) of MT can only be executed when Zn is bound to specific sites in the protein and these sites become occupied by Cd as the result of the metal interchange reaction with Cd-MT, the liver must "overproduce" Zn-MT until the proper balance between protein-bound Cd and Zn is reached to ensure Zn occupancy of the functional sites. This requirement is apparently satisfied when the ratio of Cd to Zn in MT reaches approximately 2, at which point the dual criteria of effective sequestration of all toxic Cd and Zn occupancy of the metabolically functional sites in the clusters are met.

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Copper Metallothionein from the Fungus *Agaricus bisporus*: Chemical and Spectroscopic Properties†

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ABSTRACT: The isolation and chemical characterization of the copper metallothionein from the common mushroom *Agaricus bisporus* are presented together with the complete amino acid sequence of the protein. It consists of 25 amino acids with a characteristically high cysteine content (28%) and binds 6 mol of copper per molecule. The protein reveals a high degree of sequence homology to both vertebrate metallothioneins (38.4% identity with the amino-terminal part of human metallothionein 2) and *Neurospora crassa* copper metallothionein (76.9% identity). The spectroscopic properties of *Agaricus* copper metallothionein are compared to those reported for *N. crassa* copper metallothionein [Beltramini, M., & Lerch, K. (1983) *Biochemistry 22*, 2043–2048] and suggest a very similar structure of the metal thiolate chromophore.

Metallothioneins (MTs)[1] are an ubiquitous class of low molecular weight proteins sequestering high amounts of heavy metals such as Zn, Cd, Hg, and Cu (Kägi & Nordberg, 1979). They were first isolated from equine kidney (Kägi & Vallee, 1960) and were shown to be responsible for the natural accumulation of Cd in this organ (Margoshes & Vallee, 1957). By now the amino acid sequences of MTs from a wide variety of sources including mammals, lower vertebrates, and invertebrates have been elucidated (Kägi et al., 1984).

Mammalian MTs have a typical chain length of 61 amino acid residues with the sequence positions of the 20 cysteinyl

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[1] Abbreviations: MT(s), metallothionein(s); PTH, phenylthiohydantoin; HPLC, high-pressure liquid chromatography; CD, circular dichrosism; T, trypsin; Quadrol, *N,N,N',N'*-tetrakis(2-hydroxypropyl)-ethylenediamine; R, regeneration of PTH derivative; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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