Nonoxidative Cadmium-Dependent Dimerization of Cd_7–Metallothionein from Rabbit Liver†

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ABSTRACT: The effect of free Cd(II) ions on monomeric Cd_7–metallothionein-2 (MT) from rabbit liver has been studied. Slow, concentration-dependent dimerization of this protein was observed by gel filtration chromatographic studies. The dimeric MT form, isolated by gel filtration, contains approximately two additional and more weakly bound Cd(II) ions per monomer. The incubation of MT dimers with complexing agents EDTA and 2-mercaptoethanol leads to the dissociation of dimers to monomers. The results of circular dichroism (CD) and electronic absorption studies indicate that the slow dimerization process is preceded by an initial rapid Cd-induced rearrangement of the monomeric Cd_7–MT structure. The ^{113}Cd NMR spectrum of the MT dimer revealed only four ^{113}Cd resonances at chemical shift positions similar to those observed for the Cd_4 cluster of the well-characterized monomeric $^{113}Cd_7$–MT. This result suggests that on dimer formation major structural changes occur in the original three-metal cluster domain of Cd_7–MT.

Metallothioneins (MTs)[1] constitute a class of low molecular mass proteins (6–7 kDa) characterized by their unusual amino acid composition and structure. These proteins are rich in cysteine residues and bind with a high affinity d^{10} metal ions such as Zn(II), Cd(II), Cu(I) and Hg(II). Since the biosynthesis of these proteins is induced by these metals and by certain hormones, it is believed that they play a crucial role in the physiological handling of the essential trace metals zinc and copper and in the detoxification of the nonessential metals cadmium and mercury (Kägi & Kojima, 1987; Kägi & Schäffer, 1988). Mammalian MTs contain 61 or 62 amino acids out of which 20 are conserved cysteine residues involved in the binding of seven divalent metal ions, i.e., Zn and/or Cd (Vašák & Kägi, 1983). The three-dimensional solution structures of rabbit, rat, and human liver Cd_7–MTs have been determined by two-dimensional NMR. In these structures the polypeptide-to-metal coordination bonds are identical and the polypeptide conformations show a great similarity (Frey et al., 1985; Arseniev et al., 1988; Schulze et al., 1988; Messerle et al., 1990). Moreover, there is a good agreement between the solution structure of rat liver Cd_7–MT and the recently reexamined crystal structure of Zn_2,Cd_5–MT from the same species (Robbins et al., 1991). In both the solution and the crystal structures the metals are organized into two adamantane-like metal–thiolate clusters containing three and four metal ions tetrahedrally coordinated by nine and eleven thiolate ligands, respectively.

Until now MT forms containing divalent metal ions have been considered to be principally monomeric in nature (Kägi & Schäffer, 1988). However, existence of higher molecular weight aggregates of this protein have also been reported. Thus, the formation of high molecular weight products during MT isolation under aerobic conditions has been reported (Minkel et al., 1980). Moreover, dimeric MT forms have been isolated from the liver and kidney of rabbits after administration of large quantities of Cd (Suzuki & Yamamura, 1980). Similar oligomerization of MT, induced by free metal ions, has also been observed in metal displacement experiments (Otvos et al., 1985). Since in all cases the oligomerization of MT could be reversed by treatment with 2-mercaptoethanol or dithiothreitol, an oxidative mechanism for these processes was concluded by these authors.

In this paper, the first evidence for nonoxidative dimerization of Cd_7–MT upon exposure to free cadmium ions is presented. This novel MT form contains approximately two additional weakly bound Cd(II) ions per monomer unit. The CD and ^{113}Cd NMR studies of the MT dimer suggest that major structural changes upon dimerization occur in the original three-metal cluster domain. The structural features of this novel MT form and its possible physiologic role are discussed.

Materials and Methods

Rabbit liver MT-2 was isolated from rabbits exposed to cadmium salt as described previously (Kimura et al., 1979; Kägi et al., 1974). All MT preparations were characterized by amino acid analysis (Durrum 500) and by metal analysis using atomic absorption spectroscopy (Instrumentation Laboratory, IL 157). The protein concentration was determined spectrophotometrically by measuring the absorbance of the metal-free protein (thionein) at 220 nm in 0.1 M HCl (ϵ_{220} = 47 300 M^{-1} cm^{-1}) (Bühler & Kägi, 1979). Sulfhydryl groups were quantified with 2,2-dithiopyridine, 0.2 M acetate buffer, pH 4.0, containing 1 mM EDTA (ϵ_{343} = 7060 M^{-1} cm^{-1}) (Grasseti & Murray, 1967). $^{113}Cd_7$–MT was prepared as reported (Vašák et al., 1987) using $^{113}CdSO_4$ (^{113}Cd, >95% enriched) from Harwell, England.

Gel filtration studies were performed using a Sephadex G-50 column (1.5 × 70 cm) attached to a conventional LKB chromatography unit and/or using a Superose 12 column (1 × 30 cm) attached to Pharmacia/LKB fast-protein liquid chromatography (FPLC) system. Unless otherwise stated, protein incubation and elution was performed in 20 mM potassium phosphate/0.1 M KCl buffer, pH 7.0, at 25 °C. All solutions and buffers were either argon saturated or vacuum

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[1] Abbreviations: NMR, nuclear magnetic resonance; CD, circular dichroism; FPLC, fast protein liquid chromatography; MT, metallothionein; thionein, metal-free protein.

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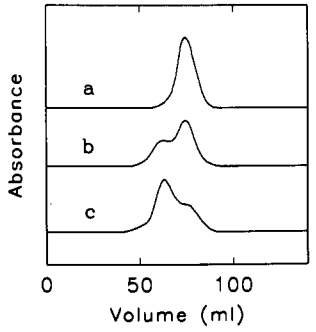

FIGURE 1: Gel filtration profiles (Sephadex G-50 column, detection at 254 nm) of (a) 65 μM Cd_7–MT and (b) the same sample incubated at 25 °C with 5 Cd equiv for 7 h and (c) 24 h. The incubation mixture and the eluting buffer contained 20 mM potassium phosphate, 0.1 M KCl, pH 7.0.

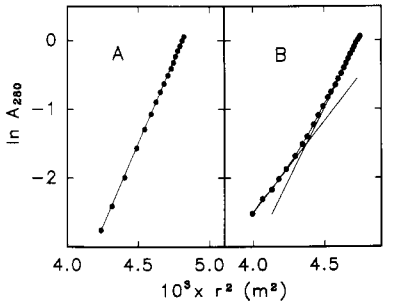

FIGURE 2: Plot of ln A_{280} vs r^2 of the equilibrium centrifugation data of (A) monomeric Cd_7–MT (rotor speed 30 000, 65 μM protein, 20 mM potassium phosphate, 0.1 M KCl, pH 7.0, 20 °C) and (B) the same sample incubated with 5 Cd equiv for 24 h (rotor speed 22 000).

degassed prior to use. Molecular weights were determined on both columns calibrated with bovine pancreatic trypsin inhibitor (BPTI) (6500), ribonuclease A (13 700), chymotrypsinogen (25 000), and ovalbumin (45 000). Kinetic data were fitted by the program Enzfitter (Biosoft, Cambridge, U.K.) using an IBM PS/2.

In ultracentrifugation experiments a Beckman L5-75B ultracentrifuge equipped with absorption optics at 280 nm was used. Molecular weights of MT forms were calculated from the conventional sedimentation equilibrium experiments (65 μM protein in 20 mM potassium phosphate, pH 7.0, containing 0.1 M KCl at 20 °C) using a plot of ln c vs r^2 (Cantor & Schimmel, 1980). The monomeric and the dimeric MT samples were run at 30 000 and 22 000 rpm, respectively. Partial specific volumes for monomeric Cd_7–MT (0.615 cm^3/g) and dimeric $(Cd_9\text{–}MT)_2$ (0.599 cm^3/g) have been calculated as described previously (Kägi et al., 1974).

The absorption measurements were performed on a Hewlett-Packard 8452A diode array spectrophotometer. A Jasco spectropolarimeter (Model J-500) interfaced with an IBM PS/2 computer was used for CD measurements. ^{113}Cd NMR spectra were recorded at 88.8 MHz on a Bruker AM-400 spectrometer. Aqueous solutions of MT (2 mM), containing 10% ^2H_2O to provide the field-frequency lock, were placed in 10-mm NMR tubes and sealed under nitrogen. Typical acquisition parameters: 60° pulse, acquisition time of 0.2 s (with ^1H broad-band decoupling), and a relaxation delay of 1.8 s (without ^1H decoupling). A line-broadening function of 40 Hz was applied prior to Fourier transformation. Chemical shifts are reported in parts per million relative to the ^{113}Cd resonance of 0.1 M $Cd(ClO_4)_2$ in ^2H_2O.

Results

Gel Filtration and Ultracentrifugation Studies. Cd_7–MT elutes from the Sephadex G-50 gel filtration column as a single chromatographic peak (Figure 1a) with an apparent molecular weight of 10 700. A similar apparent molecular weight has also been found in a number of previous chromatographic studies (Kägi et al., 1974; Vašák et al., 1984; Kägi & Kojima, 1987). In the equilibrium ultracentrifugation experiments the same sample yielded a linear plot of ln c against r^2 (Figure 2A) from which a molecular weight of 6900 was calculated. The latter value corresponds well with the analytical molecular weight of the Cd_7–MT monomer (6860) and is in agreement with results of previous ultracentrifugation studies (Kägi et al., 1974). However, incubation of the monomeric Cd_7–MT form with free cadmium ions results in a time-dependent decrease of the chromatographic peak of the monomer and the concomitant generation of a new chromatographic peak with an apparent molecular weight of 21 300 (Figure 1b,c). Ultracentrifugation studies of this sample, which displays the presence of 75% oligomer in a Sephadex G-50 gel filtration column, showed a nonlinear dependence of ln c vs r^2 (Figure 2B), reflecting molecular weight heterogeneity in the sample. The average molecular weight limits estimated from the asymptotic slopes at both ends of the curve yielded the values of 7100 and 11 400. The former value is close to analytical molecular weight of MT monomer; the latter value is somewhat lower from the expected analytical molecular weight of the dimeric $(Cd_9\text{–}MT)_2$ form (14 170) (see below). The results obtained are consistent with the concomitant presence of monomeric and dimeric MT forms in the sample thus confirming the dimeric nature of the Cd-induced oligomeric MT form. The weight heterogeneity observed in the ultracentrifugation experiment could originate either from the incomplete dimerization or from a shift in the dimerization equilibrium along the protein concentration gradient formed in the ultracentrifugation cell. The concentration dependence of the dimerization process is described below. The reason for the markedly higher apparent molecular weight of monomeric and dimeric MT forms obtained from gel filtration studies compared to expected analytical molecular weights for this form is currently not known.

Gel filtration chromatography was used to study the kinetics of Cd-induced MT dimerization. In order to shorten the rather long elution time on the Sephadex G-50 column (approximately 5 h), a Superose 12 gel filtration column attached to a FPLC apparatus was used. On this column, separation was achieved within 0.5 h (flow rate 0.4 mL/min). However, compared to the Sephadex G-50 studies the peaks of monomeric and dimeric MT were less well resolved and a 1.5 times lower dimer to monomer ratio was always obtained. Moreover, the apparent molecular weights of monomeric and dimeric MT (15 000 and 23 000, respectively) also differed from those values obtained from the Sephadex G-50 column. Attempts to improve the separation of both MT forms by employing a lower flow rate (0.15 mL/min) were unsuccessful. In this case, an even worse separation resulted and the yield of the dimer decreased. Overall, these results suggest dimer dissociation during the course of gel filtration chromatography. In further studies, the Sephadex G-50 column was used for the isolation of MT dimer.

Kinetics of MT Dimerization. In the kinetic studies of MT dimerization, 1–16 equiv of Cd were added to the Cd_7–MT (65 μM) and, at various incubation times, aliquots were applied to the Superose 12 column. The time course of dimer formation at different Cd concentrations (see Figure 3) shows that the apparent final dimer to monomer ratio depends on the metal concentration. A maximum of 50% dimer is reached with 5 and more Cd equiv added to Cd_7–MT (Figure 3, inset).

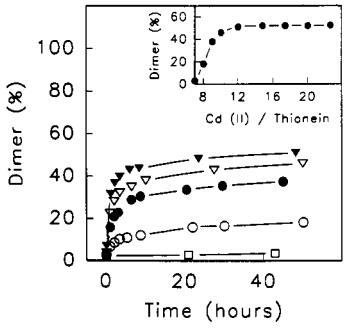

FIGURE 3: Kinetics of MT dimerization monitored by gel filtration chromatography (Superose 12 column) at the following Cd(II) to thionein ratios: (□) 7, (○) 8, (●) 9, (▽) 10, and (▼) 12. Dimer percentage (%) represents the integral ratio of dimer peak to the total area. Inset: Dependence of the final dimer percentage on Cd(II) to thionein ratios. For conditions see Figure 1.

Allowing for dimer dissociation back to monomer in the process of gel filtration chromatography (see above), samples showing 50% dimer in Superose 12 column would in effect be essentially dimeric. This conclusion is supported by the fact that a 90% yield of dimer is obtained when these samples were eluted in the presence of 0.1 mM $CdCl_2$ in the running buffer (20 mM Tris-HCl, 0.1 M KCl, pH 8.0).

Similar studies of dimer formation using a lower protein concentration (8.5 μM) in the incubation mixture revealed a substantially lower apparent yield of dimer (approximately 15% by addition of 5 Cd equiv), whereas at a higher protein concentration (170 μM) an additional minor chromatographic peak of a higher molecular weight species was observed.

Analysis of the kinetics data revealed that the MT dimerization process does not follow first-order kinetics. Due to the limited number of data points that could be obtained in the initial time period ($t < 0.5$ h), analyses using a more complex reaction scheme was not attempted.

The formation of MT dimer was also checked using the native Zn_2,Cd_5–MT form to which Cd(II) ions were added. Although in the presence of 4 equiv of Cd added to Zn_2,Cd_5–MT (65 μM) the dimeric form was also generated, the final dimer to monomer ratio was somewhat lower compared to that in similar studies using Cd_7–MT. It is well documented, however, that addition of Cd(II) ions to Zn_2,Cd_5–MT displaces the Zn(II) ions to give Cd_7–MT (Otvos & Armitage, 1981; Vašák & Kägi 1983). The resultant lower excess of Cd and, presumably, the inability of the displaced Zn to generate MT dimers may account for this effect.

Kinetics of Dimer Dissociation. MT dimer formed after a 24-h incubation of Cd_7–MT (65 μM) with 3 Cd equiv was isolated using a Sephadex G-50 column and aliquots were applied, at various time intervals, to a Superose 12 column. The rechromatography of the freshly isolated MT dimers yielded only 50% of this form. This result is consistent with the aforementioned dissociation of dimers during gel filtration. The dimer to monomer ratio decreased even further in time (Figure 4). This rather slow dissociation process is most likely caused by a perturbation of the original equilibrium in the incubation mixture, through changes in the metal and protein concentration during dimer isolation. Independent support for the existence of a dynamic equilibrium in the system studied is provided by the fact that a simple dilution of the incubation mixture containing the dimeric MT form also results in slow dissociation of dimers (data not shown).

Stability of the MT Dimer toward Complexing Agents. The stability of the dimeric MT form toward complexing agents 2-mercaptoethanol and EDTA was investigated. 2-Mercaptoethanol (1 mM) was added to the dimer sample,

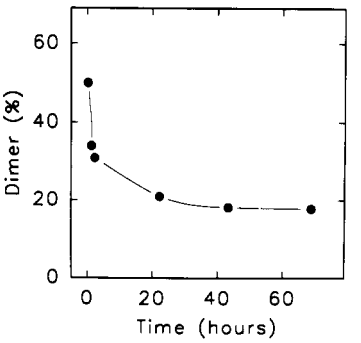

FIGURE 4: Dissociation of the isolated MT dimer (5 μM) monitored by its rechromatography on a Superose 12 gel filtration column. For conditions see Figure 1.

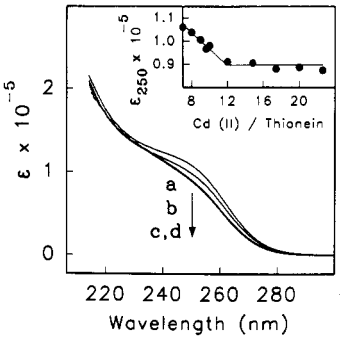

FIGURE 5: Electronic absorption spectra of Cd–MT (65 μM) at the following Cd(II) to thionein ratios: (a) 7, (b) 9.6, (c) 17.4, and (d) 22.6 in 20 mM potassium phosphate, 0.1 M KCl, pH 7.0. The spectra obtained immediately following the metal addition and after 24 h were identical. Inset: Changes in the absorption at 250 nm plotted as a function of Cd(II) to thionein ratios.

prepared by the incubation of Cd_7–MT (65 μM) with 3 equiv of Cd for 24 h. Subsequent chromatography of this sample on a Superose 12 column revealed only a single peak of monomeric MT. Since, in contrast to 2-mercaptoethanol, EDTA can remove Cd ions from Cd_7–MT (Li et al., 1980), an EDTA concentration equal to that of added Cd was used. In this case, dimer dissociation was complete within 5 h.

Overall, these results together with the previously demonstrated dissociation processes clearly demonstrate the nonoxidized nature of the Cd-induced MT dimers and highlight the importance of the additional and more weakly bound metal ions in the formation and stabilization of this form. The absence of protein oxidation in the MT dimer has also been confirmed by the quantitation of sulfhydryl groups.

Metal Ion Content of the Dimeric Cd-MT Form. The Cd content of the dimer was determined after its isolation on a Sephadex G-50 column using a procedure established for the monomeric MT form (see above). Regardless of the metal excess in the incubation mixture, a Cd to thionein ratio of 9.2 ± 0.5 was found. Note that the determined ratio relates to the monomer unit.

Electronic Absorption and Circular Dichroism Studies. The effect of excess Cd(II) ions on the electronic absorption and circular dichroism spectra of Cd_7–MT (65 μM) was examined. The spectroscopic studies were designed to differentiate between the spectral features of the monomeric and dimeric MT forms. In view of the time dependence of dimer formation, spectra were recorded immediately following metal addition and again after 24 h. Absorption spectra obtained immediately upon the metal addition (up to 16 Cd equiv) into the Cd_7–MT solution (Figure 5) show that an increasing metal concentration causes an intensity decrease of the Cd_7–MT absorption profile (up to approximately 15%) above 235 nm. This effect is most

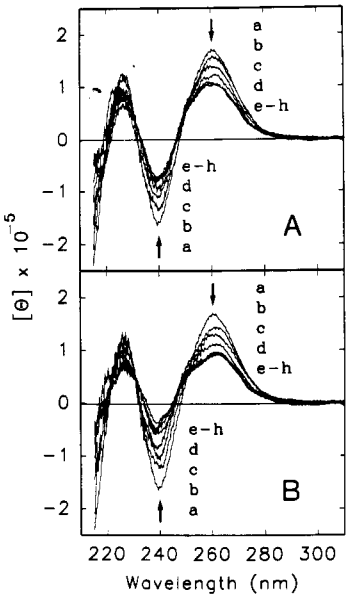

FIGURE 6: Circular dichroism (CD) spectra of Cd–MT (65 μM) at the following Cd(II) to thionein ratios: (a) 7, (b) 8, (c) 9, (d) 10, (e) 12, (f) 14.8, (g) 17.4, and (h) 22.6 recorded (A) immediately following the metal addition to Cd_7–MT or (B) after 24 h of incubation (20 mM potassium phosphate, 0.1 M KCl, pH 7.0).

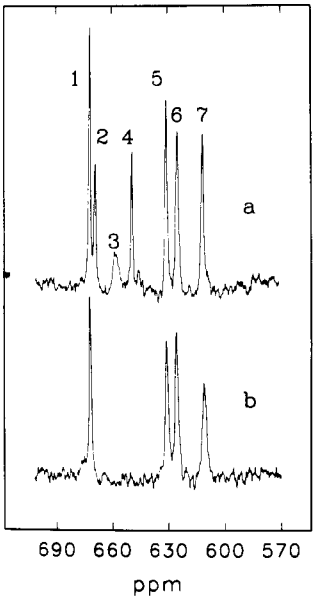

FIGURE 7: Proton-decoupled 88.8-MHz ^{113}Cd NMR spectra of rabbit liver $^{113}Cd_7$–MT: (A) monomer (10800 transients) and (B) dimer (9600 transients) in 20 mM potassium phosphate, 0.1 M KCl, pH 7.0 (25 °C). The concentration of the metal-free protein in both samples was 2 mM. The ^{113}Cd signals in (A) are numbered according to decreasing chemical shifts.

pronounced when the first 5 Cd equiv are added. Further metal additions are accompanied by only minor changes of the absorption profile. The same absorption spectra recorded after 24 h showed no apparent changes.

CD studies were conducted in parallel with absorption studies. The first set of the CD spectra recorded immediately following metal addition are shown in Figure 6A. The CD spectrum of Cd_7–MT (Figure 6A, trace a) is similar to that published (Vašák & Kägi 1983; Willner et al., 1987; Stillman et al., 1987). The first 5 Cd equiv added to Cd_7–MT induce a gradual intensity decrease of all CD bands accompanied by the development of new CD features, i.e., a shoulder at 255 nm and isodichroic points at 251 and 231 nm. Subsequent addition of up to 16 Cd equiv had no further effect on the developed CD profile. The same spectra recorded after the formation of the dimeric MT form (after 24 h) showed an alteration of the previous CD profiles with a new isodichroic point at 252 nm (Figure 6B). While only a minor CD change occurs above 245 nm, the high-energy CD band at 240 nm decreases further in intensity and the shoulder at 255 nm became more pronounced. The saturation of the spectral changes upon the addition of 5 or more equivalents of Cd suggests that under these conditions predominantly only one MT form exists. It should be noted that similar absorption and CD studies performed on 10-times-diluted samples showed substantially smaller changes in the corresponding spectral profiles. This result provides additional evidence for the dependence of MT dimerization on the protein concentration.

^{113}Cd NMR Studies. The ^{113}Cd NMR spectrum of the monomeric $^{113}Cd_7$–MT form shown in Figure 7A compares well with that published (Frey et al., 1985; Otvos et al., 1985). As previously shown the ^{113}Cd resonances 1, 5, 6, and 7 originate from the four-metal cluster and the resonances 2, 3, and 4 originate from the three-metal cluster (Otvos & Armitage 1980; Frey et al., 1985). The sample of MT dimer for the ^{113}Cd NMR measurement was prepared as follows: 80 mL of 65 μM $^{113}Cd_7$–MT was incubated for 24 h with 5 equiv of ^{113}Cd and concentrated by ultrafiltration to 2.5 mL. The final measured Cd to thionein ratio in the sample was 9.3 ± 0.4. On the basis of the gel chromatographic check, the NMR sample contained essentially the dimeric MT form. The corresponding ^{113}Cd NMR spectrum is shown in Figure 7B. In this spectrum the ^{113}Cd resonances of the Cd_3 cluster are absent and no additional resonances were observed in the spectral range between −100 and 900 ppm. The chemical shifts of the ^{113}Cd resonances of the Cd_4 cluster in both spectra are almost identical. Compared to the monomeric ^{113}Cd–MT form, the resonances 1, 5, and 7 are slightly highfield shifted (between 0.2 and 0.8 ppm), whereas the resonance 4 is downfield shifted (0.2 ppm). In addition, all resonances of the MT dimer display an increased line width (up to 50%). The latter can be accounted for by a decreased tumbling time due to the increased molecular weight of the dimeric form.

Discussion

The results of this study provide the first evidence for the generation of a nonoxidative dimeric MT form on exposure of Cd_7–MT to free Cd ions. As shown by our kinetic and spectroscopic studies, the slow dimerization process is preceded by an initial rapid rearrangement of the monomeric structure. The spectroscopic data obtained allow a number of inferences concerning the structure of the novel metal-induced MT forms.

The ^{113}Cd NMR spectrum of the MT dimer displays only four ^{113}Cd resonances at chemical shift positions very similar to that of the Cd_4 cluster of monomeric $^{113}Cd_7$–MT suggesting that this cluster organization is preserved in the dimer. Thus, major structural changes should involve the original Cd_3 cluster domain. The reason for the absence of ^{113}Cd resonances of the remaining metals bound to the MT dimer is presently unknown. Similar absence of ^{113}Cd resonances have been encountered in a number of studies on cadmium-substituted metalloproteins (Schoot Uiterkamp et al., 1980; Gettins & Coleman 1983; Coleman et al., 1979; Pan et al., 1990). In all cases, this phenomenon was attributed to a chemical exchange modulation of NMR signals with a frequency corresponding to intermediate exchange for the chemical shift difference of ^{113}Cd NMR resonances involved. Accordingly, we attribute the lack of numerous ^{113}Cd resonances in the MT dimer to a similar chemical exchange modulation. However,

more detailed NMR studies are needed to account for this observation.

In the absence of the ^{113}Cd resonances, structural features of the altered Cd_3 cluster can still be derived from the absorption and CD studies. As has been shown previously, the pronounced shoulder in the absorption spectrum of Cd_7–MT at 250 nm is due solely to CysS–Cd(II) charge-transfer bands (Kägi & Vallee, 1961). By Gaussian analysis, this shoulder has been resolved into three metal-induced bands. The lowest energy band at 250 nm was assigned to the first CysS–Cd(II) charge-transfer transition of the bridging thiolate ligands (Vašák et al., 1981). It has been shown, moreover, that in Cd(II)–thiolate complexes the transformation of terminal thiolates to bridging thiolates is accompanied by a red shift of the absorption profile (Willner et al., 1987). This is clearly not the case in our studies. Thus, the observed initial intensity decrease at 250 nm, due to a metal binding to monomeric Cd_7–MT, indicates either a decrease in the number of bridging thiolate ligands or the cancellation of excitonic interactions among some bridging thiolates (see below), or both. Accordingly, it is reasonable to assume that in the metal altered monomeric MT form nonsulfur ligands, e.g., oxygen, take part in metal coordinations. The charge-transfer bands would then be expected to occur at a higher energy (below 245 nm). Since no apparent spectral changes occur in the low-energy region of the absorption spectra (above 245 nm) upon the dimer formation, we suggest that the metal–thiolate bonds are not involved in the dimerization process.

In the CD studies the immediate Cd-induced changes develop isodichroically suggesting a transformation of the original Cd_3 cluster of Cd_7–MT into a new distinct monomeric MT structure. In the CD spectrum of Cd_7–MT, the oppositely signed CD bands at (+)260 and (−)240 nm with a cross-over point at 249 nm originate from the excitonic couplings of the bridging thiolate transitions (Willner et al., 1987; Stillman et al., 1987). Thus, the marked decrease in the magnitude of the (+)260-nm CD band of Cd_7–MT on metal binding suggests a perturbation of the bridging thiolates. The comparable magnitude of the persisting CD band at 260 nm to that of the isolated Cd_4 cluster domain (Stillman et al., 1987; Kull et al., 1990) indicates that the excitonic interactions within the original Cd_3-cluster in the metal-altered monomeric MT structure are strongly perturbed. This conclusion is supported by the occurrence of a shoulder at 252 nm in the CD spectrum, which closely resembles the position of the first charge-transfer band of the bridging thiolates (at 250 nm) in the corresponding absorption spectrum (Vašák et al., 1981; Willner et al., 1987). The CD spectra show additional changes on dimer formation such as a new isodichroic point at 252 nm and further smaller changes in the already developed CD bands. These results suggest that in the MT dimer the developed metal–thiolate cluster structures of the monomer do not change dramatically.

It is interesting to note that in the recently reexamined crystal structure of rat liver Zn_2,Cd_5–MT a dimeric arrangement of two MT molecules in a head to tail orientation has been found (Robbins et al., 1991). However, in all MT species containing seven divalent metal ions no dimeric structure has been found in solution. As shown by our results, additional metal binding leads to the formation of relatively stable MT dimers in solution. The forces stabilizing this dimeric form are at present unknown. Besides common forces stabilizing dimeric proteins, e.g., electrostatic and hydrophobic interactions and hydrogen bonds, a metal bridge(s) may also contribute to the stability of the MT dimer. In view of our results the formation of a metal–thiolate bridge(s), found in a few metal-linked dimeric proteins involved in transcriptional regulation (Frankel et al., 1988; Shewchuk et al., 1989), is unlikely. However, similar involvement of non-sulfur ligands cannot be ruled out.

Finally, a possible physiological role for the cadmium-induced dimerization of MT should also be discussed. It should be noted that MT is the only known protein in which Cd accumulates naturally and that Cd is the most potent inducer of MT biosynthesis (Kägi & Schäffer, 1988). Although considerable knowledge about the regulation of MT biosynthesis has been accumulated (Nordberg & Kojima, 1979; Palmiter, 1987; Seguin & Hamer, 1987; Seguin & Prévost, 1988), a full understanding of the mechanism as well as the identification of all proteins involved is lacking. In the light of these results, involvement of the Cd-induced MT dimers in the regulation of MT biosynthesis is conceivable. However, further studies are needed to validate this hypothesis.

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