Organization and Assembly of Metal-Thiolate Clusters in Epithelium-specific Metallothionein-4*  

Received for publication, February 22, 2006, and in revised form, March 20, 2006  
Published, JBC Papers in Press, March 23, 2006, DOI 10.1074/jbc.M601724200  

Gabriele Meloni†, Kairit Zovo§, Jekaterina Kazantseva§, Peep Palumaa§, and Milan Vasáč‡§†

From the †Department of Biochemistry, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland and §Department of Gene Technology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia

Mammalian metallothionein-4 (MT-4) was found to be specifically expressed in stratified squamous epithelia where it plays an essential but poorly defined role in regulating zinc or copper metabolism. Here we report on the organization, stability, and the pathway of metal-thiolate cluster assembly in MT-4 reconstituted with Cd²⁺ and Co²⁺ ions. Both the ¹¹¹Cd NMR studies of ¹¹¹Cd,MT-4 and the spectroscopic characterization of Co₉,MT-4 showed that, similar to the classical MT-1 and MT-2 proteins, metal ions are organized in two independent Cd₄Cys₁₁ and Cd₃Cys₉ clusters with each metal ion tetrahedrally coordinated by terminal and bridging cysteine ligands. Moreover, we have demonstrated that the cluster formation in Cd₄,MT-4 is cooperative and sequential, with the Cd₄Cys₁₁ cluster being formed first, and that a distinct single-metal nucleation intermediate Cd₃,MT-4 is required in the cluster formation process. Conversely, the absorption and circular dichroism features of metal-thiolate clusters in Cd₄,MT-4 indicate that marked differences in the cluster geometry exist when compared with those in Cd₃,MT-1/2. The biological implication of our studies as to the role of MT-4 in zinc metabolism of stratified epithelia is discussed.

Metallothioneins (MTs)² is a superfamil of low molecular mass cysteine- and metal-rich proteins or polypeptides conserved through evolution and present in all eukaryotes and certain prokaryotes (reviewed in Refs. 1 and 2). In mammals, the MT gene family consists of four subfamilies designated MT-1 through MT-4. Whereas in mouse only one member of each subfamily is present (Fig. 1), in primates a significant genetic polymorphism exists with 17 genes and pseudogenes in humans. From these 17 genes, 10 are functional, including single MT-3 and MT-4 genes (3). Mammalian MTs are composed of a single polypeptide chain of 61–68 amino acids with a conserved array of 20 cysteines and no aromatic residues or histidine. In the structurally characterized MTs (MT-1–MT-3), all cysteines are present in reduced form and are involved in the binding of seven divalent (Zn²⁺, Cd²⁺) and up to 12 monovalent metal ions (Cu⁺) forming two metal-thiolate clusters located in two independent protein domains (4–6). Although still under debate, suggested functions for mammalian MTs include homeostasis and transport of physiologically essential metals (zinc, copper), detoxification of toxic metals (cadmium, mercury), protection against oxidative stress, regulation of cell proliferation and apoptosis, and the maintenance of intracellular redox balance (7–10). Differential expression of mammalian MT isoforms is tightly regulated during development and in pathological situations (9,11). The extensively studied mammalian MT-1 and MT-2 show ubiquitous expression regulated at the transcriptional level (12). Their biosynthesis is inducible by a variety of compounds and stress conditions, such as metals, glucocorticoids, cytokines, and reactive oxygen species (9). MT-3 and MT-4 are relatively unresponsive to these inducers. MT-3 is primarily confined to the central nervous system, where it represents a major component of the intracellular Zn²⁺ pool in zinc-enriched neurons (13). Lower expression levels of MT-3 have also been reported in pancreas, kidney, reproductive tissues, and maternal deciduim. This protein exhibits growth inhibitory activity in neuronal cultures and was found down-regulated in Alzheimer disease (reviewed in Ref. 14).

The expression of the last identified mammalian metallothionein isoform, MT-4, has been found restricted to cornified, stratified, squamous epithelia, a tissue providing a protective surface on skin, footpath, tail, tongue, the upper part of the alimentary tract, and the vagina of rodents (15). Besides these tissues, the developmentally regulated MT-4 expression in maternal deciduim together with the expression of entire MT gene locus have been reported in mouse (16). Gene expression profiling by microarray hybridization of wild-type and nude mice back skin identified that the transcription factor Whn regulates the MT-4 expression together with other proteins involved in the metabolism of keratin (17). However, the question of whether MT-4 is involved in copper or zinc metabolism in epithelia is still debated. Thus, in the recent studies on the metal binding abilities of MT-4 using heterologously expressed MT-4 in Escherichia coli with zinc, cadmium, and copper in combination with the in silico protein sequence analyses, the copper binding nature of MT-4 has been suggested (18). On the other hand, based on the highly regulated and specific expression pattern of MT-4 in stratified squamous epithelium, the documented switch in expression of MT-1 in the basal layer to MT-4 expression in the next layer during differentiation of this tissue and the zinc content of the isolated protein, a special role of MT-4 in the regulation of zinc-dependent keratinocyte differentiation or in the regulation of proteolytic processing of keratins has also been suggested (15). Moreover, MT-4 expression protects cell culture against cadmium toxicity, and its expression is induced in the upper stomach of mice exposed to high zinc levels in drinking water. These observations suggest that MT-4, similar to MT-1 and MT-2, may be involved in the regulation of zinc levels and cadmium detoxification (15). However, the metal binding specificity of MT-4 is difficult to assess, as to date, only limited information regarding the metal binding properties of MT-4 containing divalent metal ions is available.

The presented studies were conducted with the aim of gaining insight into the metal binding properties and the structural features of MT-4, which are likely responsible for its function in zinc metabolism. In our studies, we have replaced the spectroscopically silent Zn²⁺ ions in the mouse Zn₇,MT-4 structure by the Cd²⁺ and Co²⁺ ions and subjected
these metal derivatives to detailed spectroscopic investigations. In the past, both Cd$^{2+}$ and Co$^{2+}$ ions have proven to be useful probes for zinc binding sites in MTs, affording a wealth of structural information on the native protein (6,19–21). From the two-dimensional [113$^{105}$Cd,$^{115}$Cd] COSY of 113$^{105}$Cd-MT-4, evidence for two distinct metal-thiolate clusters (i.e. a 3-metal and a 4-metal cluster) in this protein was obtained. Further studies on Cd$_7$MT-4 and Co$_7$MT-4 by electronic absorption, CD, and MCD spectroscopy and by mass spectrometry of partially and fully metal-loaded MT-4 afforded us information regarding the coordination geometry of the metal sites, the pathway of cluster assembly, and their stability. The results are discussed in comparison with information available on the well characterized mammalian MT-1/2 isoforms.

**EXPERIMENTAL PROCEDURES**

**Materials**—Media for protein expression were purchased from BD Biosciences. All other standard reagents were purchased of the highest purity available from common commercial sources.

**Construction of the MT-4 Expression Vector**—The pBluescript plasmid containing the coding sequence of mouse MT-4 was received from Prof. R. Palmiter, University of Washington. The expression plasmid encoding MT-4 was constructed from the cloning vector pET24d (Novagen) carrying a T7 promoter. The T7 tag sequence was eliminated (Woburn, MA).

**Preparation and Characterization of Cd$^{2+}$MT-4 Samples for Spectroscopic Titration**—The apo-form of MT-4 was generated by the method of Vašák (23), and fully Cd$^{2+}$- or $^{113}$Cd$^{2+}$-loaded MT-4 was prepared by reconstitution (23). Metal-to-protein ratios were determined using a small aliquot of the sample. The metal concentration was determined by flame atomic absorption spectrometry (SpectrAA-110, Varian Inc.) and that of the protein via sulfonometry (SpectrAA-110, Varian Inc.).

**Preparation and Characterization of Ko$^{2+}$-MT-4**—The solutions used in Ko$^{2+}$ reconstitution were rendered oxygen-free by five freeze-pump-thaw cycles on a vacuum line. The pH was checked using a small aliquot of the sample as described above. In the glove box, samples were transferred into 1 or 0.1 cm cap-sealed cuvettes for spectroscopic measurements.

**Preparation of CdMT-4 Samples for Spectroscopic Titration**—All solutions used in the Cd$^{2+}$ titration experiments were rendered oxygen-free by three freeze-pump-thaw cycles on a vacuum line and all samples prepared in a nitrogen-purged glove box. Individual apo protein samples (9 $\mu$L) in 0.1 M HCl were titrated with increasing Cd$^{2+}$ equivalents (1–8 equivalents). Subsequently, the pH value was adjusted with metal-free 1 M Tris base to 7.2. The final protein sample was contained in 10 mM Tris/HCl, 20 mM NaCl, pH 7.2. In the glove box, samples were transferred into a 1-cm cap-sealed cuvette for spectroscopic measurements.

**Preparation of CdMT-4 Samples for ESI-MS Titration**—A stock solution of apoMT-4 (100 $\mu$L) in 10 mM HCl was rendered oxygen-free by three freeze-pump-thaw cycles on a vacuum line. To 200-$\mu$L aliquots of this solution increasing equivalents (from 0 to 7 equivalents) of a stock solution of CdCl$_2$ (8.4 $\mu$L) were added. For ESI-MS analysis, samples were diluted with 20 mM NH$_4$OH (200 $\mu$L), 0.02% CH$_3$COOH, and 50% CH$_3$CN (800 $\mu$L) to a final protein concentration of 10 $\mu$L. Samples were infused through a fused silica capillary (inner diameter, 75 $\mu$m) at a flow rate of 0.5 $\mu$L/min into a nano-ESI-MS quadrupole time-of-flight Ultima API mass spectrometer (Micromass). Electrospray PicoTIPS (inner diameter, 30 $\mu$m) were obtained from New Objectiv (Woburn, MA). MS spectra were recorded in positive mode at a capillary exit voltage of 2.1 kV, cone voltage of 50 V, and RF lens energy of 50 V. Mass spectra were deconvoluted using the MaxEnt 1 software (Micromass, UK).

**Determination of Apparent Cd$^{2+}$ Binding Constant by Photometric pH Titration**—The apparent binding constants of Cd$^{2+}$-MT-4 at pH 7.0 was determined as previously described by Kägi and co-workers (25, 26) using the adapted expression of Wang et al. (27). Briefly, the release of Cd$^{2+}$ from Cd$^{2+}$-MT-4 was achieved by lowering the pH value by the addition of increasing amounts of oxygen-free 1 M HCl. Prior to measurements, a stock solution of Cd$_7$MT-4 was diluted to a final concentration of 5 $\mu$L in 10 mM Tris/HCl, 20 mM NaCl, pH 8.0, and rendered oxygen-free by three freeze-pump-thaw cycles on a vacuum line. The pH titration was performed on independent samples prepared in a nitrogen-purged glove box. Metal release was followed by recording absorption spectra between 320 and 210 nm in a sealed 1-cm cuvette. The pH was determined immediately after spectra recording using a microelectrode. The degree ($D$) of Cd$^{2+}$ release is illustrated by plotting the pH values against the percentage of metal dissociation described by

$$D = (A_{250} - A_{250,pH=1.5})/(A_{250,pH=8} - A_{250,pH=1.5}),$$

where $A_{250}$ is the absorbance at 250 nm of the Cd$^{2+}$-thiolate complex at different pH values and $A_{250,pH=8}$ and $A_{250,pH=1.5}$ represent the corresponding absorbance of Cd$_7$-MT-4 and the apoprotein, respectively. In the calculation of the apparent Cd$^{2+}$ binding constant, the $K_D$ values of the cysteine side chains were assumed to be equal to those reported for rabbit MT-1/2 isoforms ($K_D = 8.9$) (25).

**Spectroscopic Measurements**—UV-visible absorbance spectra were recorded on a Cary 3 spectrophotometer (Varian). CD and MCD measurements were performed using a Jasco (Model J-810) spectropolarimeter equipped with a 1.5-tesla electromagnet for room temperature MCD measurements. A 1-cm quartz cuvette was used for Cd$_7$MT-4.
Metal-Thiolate Clusters in Metallothionein-4

measurements and a 1- or 0.1-cm quartz cuvette for Co-MT-4 measurements. The CD spectra are expressed as molar ellipticity [θ] in units of degrees dmol⁻¹ cm², and the MCD spectra are expressed as [θM] in units of degrees dmol⁻¹ cm² tesla⁻¹.

110.9-MHz one-dimensional ¹¹³Cd NMR spectra of ¹¹³Cd-MT-4 (1.7 mM) in 20 mM Tris/HCl and 40 mM NaCl, were recorded on a Bruker DRX-500 spectrometer at 293 K using an inverse-gated broad band proton decoupling, a 34.483-Hz spectral width, a 2.2-s acquisition time, and a 4.2-s pulse repetition rate (averaging 6000 free induction decays/spectrum). The two-dimensional [¹¹³Cd-¹¹³Cd] COSY spectrum of ¹¹³Cd-MT-4 (7 msi) was acquired using the standard COSY sequence in phase-sensitive mode with proton decoupling during acquisition. The evolution period t₁ was varied in 207 increments from 0 to 10.5 ms covering a spectral width in the indirect (¹¹³Cd) dimension of 9.8 kHz. A total of 128 transients were accumulated for each value of t₁. Chemical shifts are reported in parts/million with respect to ¹¹³Cd resonance of the external standard 0.1 M Cd(ClO₄)₂ in ²H₂O. The NMR samples contained 20% ²H₂O to provide the field frequency lock and were measured in 5-mm NMR tubes.

RESULTS AND DISCUSSION

Expression and Purification of MT-4—As described under “Experimental Procedures,” MT-4 was expressed and purified as the zinc protein. The presence of a single mass peak of 6275.5 Da (calculated mass 6276.5 Da) in the ESI-M5 spectrum of metal-free MT-4 established the correctness of the recombinant protein. The cadmium-containing MT-4 was generated by the method of metal reconstitution as described above. The ¹¹³Cd-reconstituted ¹¹³Cd-MT-4 revealed a molecular mass of 7052.1 Da, which is in agreement with the calculated mass for MT-4 containing seven ¹¹³Cd ions. The analytical gel filtration experiments revealed monomeric protein with an apparent molecular mass of ~20 kDa (data not shown). The increased apparent molecular masses have also been reported for MT-1/2 and attributed to the non-globular shape of these molecules (22).

One- and Two-dimensional ¹¹³Cd NMR Characterization of ¹¹³Cd-MT-4—¹¹³Cd NMR proved to be a powerful tool in the investigation of the nature of metal binding sites and their organization in Cd-MTs (19, 20). The ¹¹³Cd NMR experiments have been performed at 110.9 MHz and at 293 K. The one-dimensional ¹¹³Cd NMR spectrum of ¹¹³Cd-MT-4 shows seven major ¹¹³Cd signals at 667, 666, 661, 659, 640, 630, and 599 parts/million, corresponding to seven distinct metal binding sites. The ¹¹³Cd resonances have been numbered from 1 to 7 in the order of decreasing chemical shifts (Fig. 2A). The chemical shift positions of the seven major resonances of ¹¹³Cd-MT-4, which are very similar to those reported for other mammalian ¹¹³Cd-MTs (20), and the presence of ¹¹³Cd-¹¹³Cd spin splitting indicate that the seven Cd²⁺ sites are organized in a cluster structure(s) in which both bridging and terminal thiolate ligands participate in metal binding.

Besides these major resonances, an additional four low intensity ¹¹³Cd resonances marked with an asterisk are also discerned (Fig. 2A). We found that, in different NMR samples, their intensities varied between 15 and 30% compared with those of the major ¹¹³Cd signals and that the overall ¹¹³Cd NMR profile was unaffected by a temperature increase from 293 to 323 K. The latter indicates that these resonances do not originate from a different cluster conformation. In addition, analytical gel filtration experiments performed after each NMR run also showed, besides the major chromatographic peak of a monomeric species, a peak of dimers (15–30%) that was formed at millimolar protein concentrations required in the NMR studies (data not shown). Because no dissociation of these dimers occurred after their rechromatography, this suggests that the dimers are linked through disulfide bond(s).

Two-dimensional [¹¹³Cd-¹¹³Cd] COSY has been used to elucidate metal cluster organization in the previously studied MT isoforms (19, 28). Therefore, to investigate the topology of the Cd-thiolate clusters in Cd-MT-4, the two-dimensional [¹¹³Cd-¹¹³Cd] COSY spectrum has been recorded at 293 K. In the spectrum analysis, only ¹¹³Cd connectivities among the major ¹¹³Cd signals of the monomer were considered. The two-dimensional plot of ¹¹³Cd-MT-4 COSY spectrum (Fig. 2B) on a “noise-free” level clearly shows the presence of five strong cross-peaks and an additional three weaker and partially resolved cross-peaks. Based on ¹¹³Cd connectivities, the topology of both metal clusters was determined (Fig. 2C). The selective association of the strong five cross-peaks with resonances 2, 5, 6, and 7 and the three weaker with resonances 1, 3, and 4 partitioned the seven ¹¹³Cd signals into two independent linkage groups. Thus, the seven metal binding sites in the protein are organized in two independent clusters, i.e. a 4-metal cluster (resonances 2, 5, 6, 7) and a 3-metal cluster (resonances 1, 3, 4). It may be
noted that the observed $^{113}\text{Cd}$ connectivities among the four low intensity $^{113}\text{Cd}$ resonances of the dimers marked with the asterisk are in line with a slightly altered 4-metal cluster in this species. Considering that all of the cysteine residues in monomeric Cd$_7$MT-4 are involved in metal binding (see also next paragraph), the NMR data suggest the presence of the Cd$_4$Cys$_{11}$ and Cd$_3$Cys$_9$ clusters in which five and three cysteine thiolates act as bridging ligands, respectively. The identical cluster topologies have also been found in the mammalian Cd$_7$MT-1/2 (29, 30). Moreover, in view of the conserved array of 20 cysteine residues in all mammalian MTs, the Cd$_4$Cys$_{11}$ cluster of MT-4 would be located in the C-terminal $\alpha$-domain and the Cd$_3$Cys$_9$ cluster in the N-terminal $\beta$-domain.

Electronic Absorption, CD, and MCD Characterization of Co$_7$MT-4 and Cd$_7$MT-4—Because no direct information regarding the geometry of the metal binding sites and the clusters can be obtained from $^{113}\text{Cd}$-$^{113}\text{Cd}$ COSY experiments, further spectroscopic investigations of the Co$_7$MT-4 and Cd$_7$MT-4 derivatives were conducted. To unambiguously establish the coordination geometry and ligation of metal binding sites in MT-4, the Co$_7$MT-4 derivative was characterized by electronic absorption and room temperature MCD spectroscopy (Fig. 3). The visible region of the corresponding absorption spectrum shows a d-d profile characterized by two broad bands with maxima at 744 and 693 nm and a poorly resolved shoulder at -610 – 620 nm. The former two bands have their respective molar absorbances of 2700 and 2950 M$^{-1}$ cm$^{-1}$, i.e. \( \varepsilon = 386 \text{ M}^{-1} \text{ cm}^{-1} \) and 421 M$^{-1}$ cm$^{-1}$/Co$^{2+}$ bound to protein, respectively. The position and the intensity of the d-d features are typical of tetrahedral tetrathiolate coordination reported for inorganic model complexes and other protein cobalt derivatives with cysteine thiolate ligands. The resolved d-d pattern can be assigned to the spin-allowed $v_3$ $[^4\text{A}_2 \rightarrow ^4\text{T}_2(\Pi)]$ transition, as already described for rabbit liver MT-1 (31). Evidence for tetrahedral tetrathiolate Co$^{2+}$ coordination is provided by the MCD spectrum of Co$_7$MT-4. The low energy region of the MCD spectrum shows a strong negative band at 743 nm with a pronounced shoulder at around 690 nm. In addition, a weak positive band at 628 nm and a weak negative band at 573 nm were observed. A similar overall pattern has been theoretically predicted and observed in a number of inorganic tetrahedral and pseudotetrahedral model compounds and in other Co$^{2+}$ protein derivatives possessing these coordination geometries (Ref. 31 and references therein). Splitting of the $v_3$ transition in the absorption spectrum into three components with an energy separation of \( \sim 2000 \) and 1000 cm$^{-1}$ is larger than that expected from spin-orbit coupling alone. This and the molar extinction coefficient of the most intense band at 693 nm \( \varepsilon = 421 \text{ M}^{-1} \text{ cm}^{-1}/\text{Co}^{2+} \) is in agreement with a pseudotetrahedral symmetry of the sites \( \varepsilon > 250 \text{ M}^{-1} \text{ cm}^{-1} \) (32). Evidence for cobalt-sulfur coordination was obtained from the high energy part of the absorption spectrum, where a strong absorption band at 325 nm \( \varepsilon = 20200 \text{ M}^{-1} \text{ cm}^{-1} \) and a shoulder \( \sim 390 \) nm was observed. The position of the CysS-Co$^{2+}$ LMCT transition at 325 nm and its intensity \( \varepsilon = 1010 \text{ M}^{-1} \text{ cm}^{-1}/\text{CysS-Co}^{2+} \) bond are consistent with 20 Cys residues being involved in metal binding (33).

To learn more about the cluster geometry in Cd$_7$MT-4, its electronic absorption, CD, and MCD spectra were examined (Fig. 4). The electronic absorption spectrum of Cd$_7$MT-4 (Fig. 4A) shows a characteristic
Metal-Thiolate Clusters in Metallothionein-4

FIGURE 5. Determination of apparent Cd\(^{2+}\) binding constants. The metal dissociation from Cd\(_7\)MT-4 with decreasing pH values was monitored through the decrease of the CysS-Cd\(^{2+}\) LMCT absorption band at 250 nm presented in Fig. 3A. Conditions: 5 \(\mu\)M Cd\(_7\)MT-4 in 10 mM Tris/HCl, 20 mM NaCl, pH 8.0, was titrated with increasing amounts of 1 M HCl. Degree of metal release \(D = (I_{250} - I_{250,\text{Cd}^{2+}})/I_{250,\text{Cd}^{2+}}\) is plotted as a function of decreasing pH values. For details, see “Experimental Procedures.”

shoulder at \(\sim 250\) nm, a feature commonly found in mammalian Cd\(_7\)MTs. By analogy, we assign the underlying metal-induced bands to CysS-Cd\(^{2+}\) LMCT transitions (34). It should be noted that metal-free MT-4 (apoMT-4) does not show appreciable absorption above 230 nm due to the absence of aromatic amino acids and histidine. The molar extinction coefficient at 250 nm of \(\sim 100,000\) M\(^{-1}\) cm\(^{-1}\) reveals a value of 5000 M\(^{-1}\) cm\(^{-1}\)/CysS–Cd\(^{2+}\) bond. The calculated value is closely similar to that reported for the CysS–Cd\(^{2+}\) bond in a number of Cd\(^{2+}\)-substituted metalloproteins (35), confirming that all 20 cysteines in MT-4 are involved in metal binding. The corresponding MCD spectrum of Cd\(_7\)MT-4 (Fig. 4C) shows a biphasic profile with bands at (-)258 and (+)236 nm with the inflection point at 247 nm reported also for other mammalian Cd\(_7\)MTs (36,37). These features have been assigned to a positive A term originating from Cd\(^{2+}\) binding sites possessing a Td-type symmetry (21). However, the CD profile of Cd,MT-4 (Fig. 4B) with extrema at (+)266, (-)245, and (+)228 nm substantially differ from those reported for different members of Cd\(_7\)MT-1/2 subfamilies (21,36–38). Thus, in the structurally well characterized rabbit Cd\(_2\)MT-2 and mouse Cd\(_7\)MT-1, characteristic CD bands at (+)261, (-)241, and (+)228 nm have been reported. Moreover, it has been demonstrated that the oppositely signed low energy CD bands represent an envelope of biphatic CD bands originating from the excitationally coupled transition dipole moments of the bridging thiolate ligands within the cluster structure. This was further confirmed by the identity of the positions of the first low energy CysS-Cd\(^{2+}\) LMCT transition at 249 nm (obtained from a Gaussian analysis of the corresponding difference absorption profile) with the crossover point in the corresponding CD spectra at 250 nm (36,39). Because the CD spectra are highly sensitive to structural changes, the observed red shift of both low energy CD bands with a crossover point at 258 nm suggest marked alterations of the cluster geometry in Cd\(_7\)MT-4. This conclusion is supported by detailed analysis of the absorption and CD profiles presented in the next paragraph.

Determination of Apparent Binding Constant for Cd\(^{2+}\) Bound to MT-4—To investigate the stability of metal-thiolate clusters in MT-4, the apparent Cd\(^{2+}\) binding constants were determined through pH titration (Fig. 5). This spectroscopic method is based on the competition between protons and metals for cysteine thiolates (25,27). In very recent studies, this method has been used to compare the stability of cadmium-thiolate clusters in MT-1, -3, and -4. However, in these studies, no apparent stability constants for the MT-4 clusters were calculated (40). The decreasing intensity of the CysS-Cd\(^{2+}\) LMCT band at 250 nm on progressive acidification reflects metal release from the protein. Cd,MT-4 shows a two-step titration profile with the pH midpoint values of 4.1 and 2.9, indicating different stabilities of its two metal-thiolate clusters. Based on the absorbance decrease at the plateau between the two titration steps, the number of thiolate ligands involved in metal binding in each cluster can be derived. Because a total of 20 cysteines are involved in metal binding, the decrease of the CysS-Cd\(^{2+}\) LMCT absorption of \(\sim 0.45\) corresponding to nine cysteines can be assigned to the less stable 3-metal cluster (\(\beta\)-domain) and that of \(\sim 0.55\) to the 4-metal cluster containing 11 cysteines (\(\alpha\)-domain). The pH midpoint values for each titration step have been used for independent calculation of the apparent Cd\(^{2+}\) binding constant \(K_{\text{app},pH=7}\) of both clusters. The obtained values of \(K_{\text{app},pH=7}\) of 6.7 \(\times\) 10\(^{13}\) M\(^{-1}\) for the 3-metal cluster and of 7.5 \(\times\) 10\(^{13}\) M\(^{-1}\) for the 4-metal cluster are similar to values of 2.3 \(\times\) 10\(^{14}\) M\(^{-1}\) and 6.3 \(\times\) 10\(^{13}\) M\(^{-1}\) reported for the corresponding clusters in rabbit
Cd₄-MT-2 (27). However, compared with Cd₄-MT-2, the 3-metal cluster in Cd₇-MT-4 shows a slightly lower metal binding affinity with the metal release occurring already below pH 6 and over a wider pH range. This behavior may suggest the presence of metal binding sites with slightly different binding constants within this cluster.

**Pathway of Cluster Assembly in Cd₇-MT-4**—To gain an insight into the pathway of cluster assembly, the filling up process of apoMT-4 with Cd²⁺ has been followed by spectroscopic (electronic absorption, CD, and MCD) and spectrometric (nano-ESI-QTOF-MS) techniques. In the absorption spectra, the stepwise addition of Cd²⁺ equivalents to apoMT-4 (at pH 7.2) resulted in an incremental increase of the absorption profile (Fig. 6A). This is better documented in the difference absorption spectra of metal-bound protein from which the absorption spectrum of apoMT-4 was subtracted (Fig. 6A, inset). The difference absorption envelope of the Cd-MT-4 complexes increases monotonically until the protein saturation with seven Cd²⁺ equivalents is reached. Further addition of Cd²⁺ equivalents was without effect, confirming that, in Cd₇-MT-4, all 20 cysteines are involved in metal binding. In the corresponding CD spectra, dramatic changes as a function of the metal binding site occupation occurred (Fig. 6B). The addition of the first Cd²⁺ equivalent introduced a broad positive CD envelope with an extremum at ~240 nm. Further metal addition led to a progressive development of a multiphasic CD profile characterized by two positive ellipticity bands and an interspaced negative ellipticity band. Both the positive CD band at 228 nm and the negative at 245 nm gradually increased in intensity up to seven Cd²⁺ equivalents added. However, the low energy positive CD band reached maximum intensity already with the first four Cd²⁺ equivalents. The binding of the remaining three Cd²⁺ equivalents resulted in a progressive red shift of this CD band from 259 to 266 nm. A plot of the changes in intensity at 258 nm as a function of Cd²⁺ equivalents bound reveals a clear break point between four and five Cd²⁺ equivalents (Fig. 6B, inset). These results, together with the results presented below, indicate the progressive formation of the more stable 4-metal cluster, which is followed by the formation of the 3-metal cluster. In the corresponding MCD spectra of CdMT-4 complexes, the characteristic positive A-term signal of Cd²⁺ binding sites possessing a T₄-type of symmetry is preserved throughout the cluster formation process (Fig. 6C).

From the difference CD spectra in which the contribution of the apoprotein was subtracted a better understanding of the pathway of the 4-metal cluster formation could be obtained (Fig. 7). From the occurrence of a broad positive envelope in the first titration step and the development of two isodichroic points in the following three titration steps, it appears that initially a Cd₃-MT-4 species is formed followed by the cooperative formation of the 4-metal cluster. The presence of a nucleation intermediate Cd₃-MT-4 suggests that only a partial cooperativity exists in this cluster. This conclusion is supported by the corresponding ESI-MS titration experiments in which the co-existence of apoMT-4, Cd₄-MT-4, and Cd₇-MT-4 species is observed when less than four Cd²⁺ equivalents were added (Fig. 8). This suggests that the formation of the nucleation intermediate Cd₃-MT-4 is required to create a template for the cooperative binding of the next three metal ions. It may be noted that the monometallic nucleation intermediate has also been observed in the formation of the Cu²⁺-thiolate cluster in copper chaperone Cox17, which apopform is mainly unstructured (41).

Evidence for a full cooperativity in the 3-metal cluster formation came from the development of isodichroic points at ~217 and 239 nm upon the binding of the last three Cd²⁺ equivalents to Cd₄-MT-4 (see Fig. 6B, CD panel) and from the corresponding ESI-MS data (Fig. 8). In the ESI-MS spectra, besides the mass peak of Cd₄-MT-4, the mass peak of Cd₅-MT-4 was also discerned. The observed mass for Cd₅-MT-4 may originate from the formation of other intermediate species or from the required conditions for the ESI-MS analysis (pH = 6). However, in view of a partial metal release from Cd₅-MT-4 already at pH 6 (see “Determination of Apparent Binding Constant for Cd²⁺ Bound to MT-4”), we favor the latter effect.

**Analysis of Metal-induced Absorption and CD Profiles of Cd₂-MT-4 and Cd₄-MT-4**—As discussed above in MT-4, the identical cluster organization of tetrahedral metal binding sites exist when compared with
Metal-Thiolate Clusters in Metallothionein-4

FIGURE 9. A, circular dichroism spectra of Cd$_7$MT-4 and Cd$_4$MT-4. Shown are Gaussian analyses of the UV difference absorption spectra of Cd$_7$MT-4 versus apoMT-4 (B) and Cd$_4$MT-4 versus apoMT-4 (C). The original CD and absorption spectra are presented in Fig. 6.

CONCLUSION

Considering the results obtained in this investigation on Cd$^{2+}$ and Co$^{2+}$ binding to MT-4, it can be concluded that this protein possesses divergent metal binding properties similar to the ubiquitously expressed MT-1/2 isoforms. MT-4 is able to bind seven Cd$^{2+}$ through 20 cysteine thiolates into two separate Cd$_5$S$_{11}$ and Cd$_3$S$_9$ clusters located in two independent protein domains. All metal ions present in the clusters are tetrahedrally coordinated by both terminal and bridging thiolates. On the other hand, significant differences to mammalian MT-1/2 also exist. Thus, although the pathway of cluster formation shows a similar cooperative and sequential formation of both metal-thiolate clusters, a single metal intermediate is formed within the 4-metal cluster of MT-4 during its assembly. This feature is unique to this MT isoform. In addition, the Cd$^{2+}$ ions in the 3-metal cluster are bound with a slightly lower affinity and the filling up process of this cluster leads to substantial alterations of the cluster(s) geometry. The reason for these differences may originate from the differences between the primary structures of MT-4 and MT-1/2. Although within the MT-4 subfamily a high sequence homology conservation exists (93.4% identity between mouse MT-4 and human MT-4), a poor degree of conservation in non-cysteine amino acids is seen among MTs present in the same organism, which suggests stronger functional evolutionary constraints for this isoform. From the comparison of MT-4 and MT-1 sequences, a higher divergence for the
β-domain (3-metal cluster) than for the α-domain (4-metal cluster) of MT-4 has been observed (18). In the α-domain of MT-4, characteristic Cys-Pro and Cys-Pro-Pro peptide sequences are found (Fig. 1), which are conserved within the MT-4 subfamily. Prolines are known to introduce structural constraints due to the turns imposed on the polypeptide chain by the peptidyl–prolyl bonds and the high energy barrier for their cis-trans isomerisation. The influence of such structural constraints on the properties of the β-domain of MT-3 has been reported (37, 38). In this protein, the conserved Cys-Pro-Cys-Pro motif introduced changes in the 3-metal cluster geometry and its dynamics, features found to be essential for the biological activity of MT-3 (37, 38). The higher content of bulky amino acid and a Glu insert in position 5 in the β-domain of MT-4 together with the in silico protein sequence analyses led to the conclusion that this protein domain would be substantially more bulky than that in MT-1 (see Fig. 1). In contrast, similar analyses of the α-domain revealed that the change in the volumes would be rather small (18). These features of both protein domains in MT-4 are apparently responsible for its structure upon metal binding. Thus, although the geometry of the 4-metal cluster in Cd₄MT-4 was found similar to that of MT-1/2, the formation of the 3-metal cluster plays a major role in generating the final protein structure. Hence, to account for our data, we hypothesize that the metal filling of the bulky β-domain may lead to a mutual interaction between the β- and α-domains of MT-4, which in turn, would lead to a change in geometry, presumably in both subunits. In this case, the structural constraints imposed by the aforementioned prolines may play an important role. In the previous studies the in silico protein sequence analyses of MT-4 together with the results of heterologous E. coli expression of the protein with zinc, cadmium, or copper led to the suggestion that MT-4 differentiated toward copper thionein character, playing a role in copper metabolism in the stratified squamous epithelium (18). However, a role of MT-4 in zinc homeostasis has also been suggested (15). The latter and our data showing similar cadmium binding affinities of the metal-thiolate clusters in MT-4 and MT-1/2, overall similarities in the cluster topologies, the metal binding geometry, and the pathway of cluster assembly make the role of MT-4 in zinc metabolism in keratinocytes evenly likely. Taken together, particular structural features of MT-4 in relation to the metal homeostasis of the extremely specialized tissues in which MT-4 is expressed suggest that this protein may be involved in both zinc and copper metabolism.

Acknowledgments—We thank Dr. T. Fox (Institute of Inorganic Chemistry, University of Zürich, Switzerland) for recording the 71Cd NMR spectra and Dr. S. Chesnov (Functional Genomic Center, Zürich, Switzerland) for obtaining nano-ESI-MS spectra.

REFERENCES
1. Kägi, J. H. R. (1993) in Metallothionein III (Suzuki, K. T., Imura, N., and Kimura, M., eds) pp. 29–56, Birkhäuser Verlag, Basel, Switzerland
2. Vallee, B. L. (1995) Neurochem. Int. 27, 23–33
3. Binz, P. A., and Kägi, J. H. R. (1999) in Metallothionein IV (Klaassen, C. ed) pp. 7–13, Birkhäuser, Basel, Switzerland
4. Vašák, M., and Hasler, D. W. (2000) Curr. Opin. Chem. Biol. 4, 177–183
5. Romero-Isart, N., and Vašák, M. (2002) J. Inorg. Biochem. 88, 388–396
6. Vašák, M., and Romero-Isart, N. (2005) in Encyclopedia of Inorganic Chemistry (King, R. B., ed) 2nd Ed., pp. 3208–3221, J. Wiley & Sons Ltd., New York
7. Maret, W. (2000) J. Natr. 130, (suppl.) 14555–14585
8. Palminter, R. D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8428–8430
9. Miles, A. T., Hawksworth, G. M., Beattie, J. H., and Rodilla, V. (2000) Crit. Rev. Biochem. Mol. Biol. 35, 35–70
10. Hidalgo, J., Aschner, M., Zatta, P., and Vašák, M. (2001) Brain Res. Bull. 55, 133–145
11. Theocharis, S. E., Margeli, A. P., Klijanienko, J. T., and Kouraklis, G. P. (2004) Histopathology 45, 103–118
12. Lichtlen, P., and Schaffner, W. (2001) BioEssays 23, 1010–1017
13. Masters, B. A., Quaife, C. J., Erickson, J. C., Kelly, E. J., Froelick, G. J., Zambrowicz, B. P., Brinster, R. L., and Palminter, R. D. (1994) J. Neurosci. 14, 5844–5857
14. Sogawa, C. A., Asanuma, M., Sogawa, N., Miyazaki, I., Nakanishi, T., Furuta, H., and Ogawa, N. (2001) Acta Med. Okayama 55, 1–9
15. Quaife, C. J., Findley, S. D., Erickson, J. C., Froelick, G. J., Kelly, E. J., Zambrowicz, B. P., and Palminter, R. D. (1994) Biochemistry 33, 7250–7259
16. Liang, L., Fu, K., Lee, D. K., Sobieski, R. J., Dalton, T., and Andrews, G. K. (1996) Mol. Reprod. Dev. 43, 25–37
17. Schlake, T., and Boehm, T. (2001) Mech. Dev. 109, 419–422
18. Tio, L., Villarreal, L., Attian, S., and Capdevila, M. (2004) J. Biol. Chem. 279, 24465–24473
19. Vašák, M. (1998) Biodegradation 9, 501–512
20. Öz, G., Pountney, D. L., and Armitage, I. M. (1998) Biochem. Cell Biol. 76, 223–234
21. Stillman, M. J. (1992) in Metallothioneins (Stillman, M. J., Shaw, C. F. I., and Suzuki, K. T., eds) pp. 55–127, VCH Publishers, Inc., New York
22. Faller, P., Hasler, D. W., Zerbe, O., Klauera, S., Winge, D. R., and Vašák, M. (1999) Biochemistry 38, 10158–10167
23. Vašák, M. (1991) Methods Enzymol. 205, 452–458
24. Pedersen, A. O., and Jacobsen, J. (1980) Eur. J. Biochem. 106, 291–295
25. Vašák, M., and Kägi, J. H. R. (1983) in Metal Ions in Biological Systems (Sigel, H., ed) Vol. 15, pp. 213–273, Marcel Dekker Inc., New York and Basel, Switzerland
26. Kägi, J. H. R., and Vallea, B. (1961) J. Biol. Chem. 236, 2435–2442
27. Wang, Y., Mackay, E. A., Kurasaki, M., and Kägi, J. H. R. (1994) Eur. J. Biochemistry 225, 449–457
28. Ottos, J. D., Engeseth, H. R., and Wehrli, S. (1985) Biochemistry 24, 6735–6740
29. Braun, W., Vašák, M., Robbins, A. H., Stout, C. D., Wagner, G., Kägi, J. H. R., and Wüthrich, K. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10124–10128
30. Zanunger, K., Öz, G., Ottos, J. D., and Armitage, I. M. (1999) Protein Sci. 8, 2630–2638
31. Vašák, M., Kägi, J. H. R., Holmqquist, B., and Vallea, B. L. (1981) 20, 6659–6664
32. Bertini, I., and Luchinat, C. (1983) in Metal Ions in Biological Systems (Sigel, H., ed) Vol. 15, pp. 101–156, Marcel Dekker Inc., New York and Basel, Switzerland
33. Vašák, M. (1980) J. Am. Chem. Soc. 102, 3953–3955
34. Vašák, M., Kägi, J. H. R., and Hill, H. A. O. (1981) Biochemistry 20, 2852–2856
35. Henehan, C. J., Pountney, D. L., Zerbe, O., and Vašák, M. (1993) Protein Sci. 2, 1756–1764
36. Willner, H., Vašák, M., and Kägi, J. H. R. (1987) Biochemistry 26, 6287–6292
37. Hasler, D. W., Jensen, L. T., Zerbe, O., Winge, D. R., and Vašák, M. (2000) Biochemistry 39, 14567–14575
38. Romero-Isart, N., Jensen, L. T., Zerbe, O., Winge, D. R., and Vašák, M. (2002) J. Biol. Chem. 277, 37023–37028
39. Willner, H., Bernhard, W. R., and Kägi, J. H. R. (1992) in Metallothioneins (Stillman, M. J., Shaw, C. F. I., and Suzuki, K. T., eds) pp. 128–143, VCH Publishers, Inc., New York
40. Cai, B., Zheng, Q., and Huang, Z. X. (2005) Protein J. 24, 327–336
41. Palmaua, P., Kangur, L., Voronova, A., and Stillard, R. (2004) Biochem. J. 382, 307–314