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THE COMPLEXATION OF APO-METALLOTHIONEIN WITH CADMIUM ION AND ITS CONFORMATION STUDY

Deying Chu, Yu Lin, Binggen Ru and Ruilin Liu*

Department of Chemistry, Peking University, Beijing, 100871
*Department of Biology, Peking University, Beijing, 100871, P. R. China

Abstract

The circular dichroism was used to study the complexation of two isoforms of rabbit liver apo-metallothioneins (apo-MT1 and apo-MT2) with Cd^{2+} ion and the influence of Cd^{2+} ion on the conformation of reconstituted MTs. The stability of sulfhydryls of apo-MT has been investigated at the room temperature in the presence of air. The reconstitutions of apo-MT1 with Cd^{2+} ion were carried out at *pH* 4.71 (stable state) and *pH* 7.9 (with 90% sulfhydryls oxidated) respectively. It was found that the characteristic CD band at 257 nm(+), 238 nm(−), 226 nm(+) of reconstituted MT with Cd^{2+} ion was the same as native MT at *pH* 4.71, however only one peak at 243 nm(+) appeared on the CD spectra at *pH* 7.9 which arose from mononuclear complexes with four separated thiolate ligands per Cd^{2+} ion.

The CD spectra of apo-MTs + 7 eq Cd^{2+} system were measured at various *pH* values. It was found that the peak at 256 nm of apo-MT1 binding Cd^{2+} ion split into two small peaks at *pH* between 2.42 and 3.02, and became one peak at *pH* 3.32, while the shapes of Cd peaks of apo-MT2 binding Cd^{2+} ion did not change with *pH*, indicating that the binding sites and pathway of apo-MT1 binding Cd^{2+} ion were different from those of apo-MT2. A possible mechanism was suggested.

Keywords: cadmium, circular dichroism, complexation, conformation, metallothionein

Introduction

It has been suggested that metallothionein provides metals such as zinc and copper where and when needed for whatever role [1] in organism. Its other functions have been found recently including as a free radical scavenger [2], anti-radioactivity [3], detoxication of heavy metals [4] and its potential ability to afford protection against platinum when the later is administered as the chemotherapeutic agent *cis*-dichlorodiamine-Pt(II)(*cis*-DDP) for cancer [5].

For nearly 20 years, scientists have been making attempts to discover the mechanism from the aspect of structure and function, and explored its potential applications. Kaegi pointed out that MT/apo-MT pair, as a donor and acceptor of metal ions, serving as a fast-responding biosynthetically tunable metal-bufer-

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ing system, and playing a key role in modulating the concentration of free zinc ion cellular functions need [6]. In order to reveal molecular reaction mechanism of biological functions of MT/apo-MT pair, authors believe that it is necessary to study the complexation behaviour of apo-MTs with metal ions and their influence on normal conformation of MT. In the process of metal metabolism, which are concerned in releasing metal ions from MT and binding metal ions of apo-MT, structure of MTs must be changed dynamically. Authors try to understand how the complexation of apo-MTs with metal ions affect the MT conformation making up and breaking down by a series of studies.

A comparative test was carried out in this paper about two isoforms of rabbit liver metal free metallothionein (apo-MT1 and apo-MT2) complex with sufficient Cd^{2+} ion. Different isoforms of metallothionein have been suggested that they may have different biological functions [1, 3]. Authors have reported the properties of mouse native MT2 and rabbit liver MT1 [7], and the comparative experiments of rabbit liver apo-MT1 and apo-MT2 complexing with Hg^{2+} ion were carried out [8]. It has never been reported on comparative tests of physicochemical properties of MT isoforms.

Material and method

Rabbit liver metallothionein was isolated from rabbit induced with $CdCl_2$. The mixture of MTs was eluted on a Sephadex G-25 column at *pH* 8.6, then MT1 and MT2 were separated by DEAE-Sepharose Fast Flow (Pharmacia) ion-exchange column eluted with a linear gradient of 0.01–0.35 *M* Tris HCl at *pH* 8.6. Removing metal ion on a Sephadex G-25 column at *pH* 2 apo-MT-1 and -2 was undertaken. Using Ellman reagent [9], the concentrations of sulfhydryl groups of apo-MTs were determined by absorbance at 412 nm, (the standard curve was measured with cysteine (Sigma), $\varepsilon_{412}=13600 L\ mol^{-1}cm^{-1}$). Apo-MT concentrations were determined by absorbance at 220 nm, ($\varepsilon_{220}=47300 L\ mol^{-1}cm^{-1}$). The two results gave a stoichiometric ratio of 20 sulfhydryls per protein molecule with errors within 5–10%.

The CD spectra of MTs reconstituted by apo-MT with Cd^{2+} ion were recorded on a Jasco J-500C automatic recording spectropolarimeter (Shimadzu, Japan). All experiments were carried out at room temperature and in the presence of air with stirring.

The average number of sulfhydryls per MT molecule at different *pH* was determined first (shown in Table 1). The conditions of reconstitutions of apo-MT1 with Cd^{2+} ion were chosen at two typical *pH* values, they were *pH* 4.71 (sufhydryl was stable) and 7.9 (sulfhydryl was easily oxidated).

1) The *pH* of apo-MT1 solution was adjusted from 2 to 4.71 by addition of 0.7 $mol \cdot l^{-1}$ NaAc, then the number of sulfhydryls per MT molecule measured

Table 1 *pH* dependency of sulfhydryl number of apo-MT2 ($c=2.8\times10^{-5}$ mol·l^{-1})*

pH value	1.69	1.75	2.53	3.65	4.67	5.49	6.34	7.08	7.97	9.01
remanent sulfhydryls (%)	100	100	97	100	86	78	57	19	11	13.1

* error 10%

was 20. Adding $Cd(NO_3)_2$ (0.0342 mol·l^{-1}) into apo-MT1 solution ($c=0.95\times10^{-5}$ mol·l^{-1}) successively with 1 equivalent (eq) each time, their CD spectra were shown in Fig. 1 (a quartz cell with a pathlength of 1.0 cm was employed; sensitivity; 5 m°/cm; chart speed: 5 cm/min; time constant: 1 s; wavelength expansion: 10 nm/cm; the scan range of wavelength was from 290 to 200 nm). Being dealt with computer, the Cd-induced differential CD spectra were obtained by subtracting the spectrum 1 of apo-MT1 from those induced by addition of Cd^{2+} ion (Fig. 2).

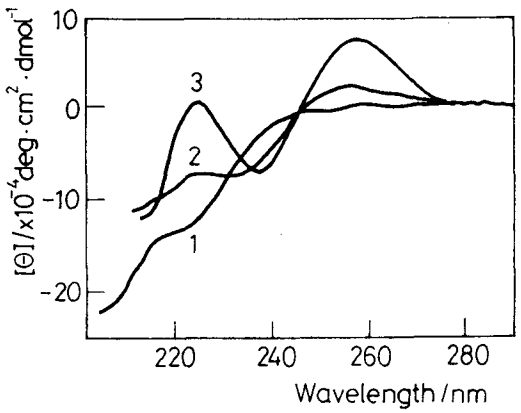

Fig. 1 The CD spectra of rabbit apo-MT1 with binding of successive equivalents (eq) of Cd^{2+}, $c_{apo\text{-}MT1}=0.95\times10^{-5}$ mol·l^{-1}, *pH* 4.71. 1) apo-MT; 2) adding 2 eq Cd^{2+}; 3) adding 7 eq Cd^{2+}. [θ]-molecular ellipticity; molecular weight of MT: 6600

2) When apo-MT ($c=1.83\times10^{-5}$ mol·l^{-1}) solution was adjusted from *pH* 2 to *pH* 7.9 by addition of K_3PO_4 (2 mol·l^{-1}), the number of sulfhydryls was measured. Adding 0.2 eq Cd^{2+} each time the CD spectra were recorded until they changed no longer (Fig. 3) at *pH* 7.9.

3) The CD spectra of reconstituted MTs by apo-MT1 and apo-MT2 with sufficient Cd^{2+} ion were recorded at various *pH*. To apo-MT1 solution ($c=1.63\times10^{-5}$ mol·l^{-1}), was added about 7.5 eq Cd^{2+} ion at *pH* 2, then the *pH* value was adjusted carefully by adding NaOH (3 mol·l^{-1}) with stirring. The CD spectra were measured (0.5 cm quartz cell; sensitivity: 2 m°/cm; chart speed: 5 cm/min; time constant: 1 s; wavelength expansion: 10 nm/cm; scan range: 320–190 nm) at *pH* 2.01, 2.42, 2.73, 2.85, 3.02, 3.32, 3.75, 4.12 as shown in

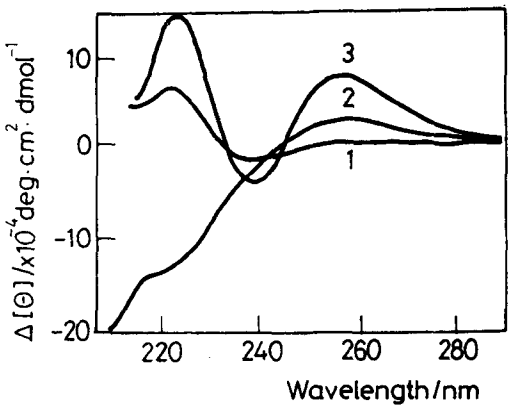

Fig. 2 The differential CD spectra of apo-MT1 subtracted from CD spectra of that with adding of Cd^{2+}. 1) apo-MT1; 2) Δ[θ] obtained by subtracting the spectrum 1 from 2 in Fig. 1; 3) Δ[θ] obtained by subtracting the spectrum 1 from 3 in Fig. 1

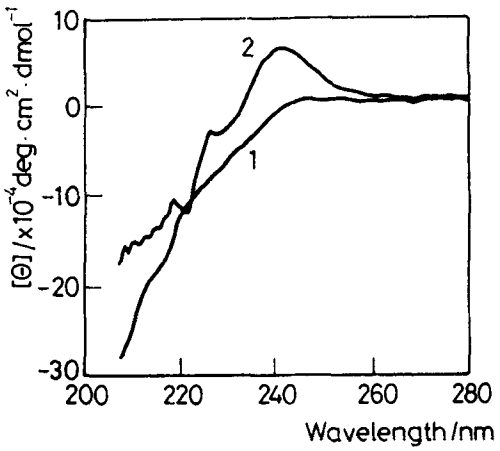

Fig. 3 The CD spectra of rabbit apo-MT1 adding 0.7 eq Cd^{2+} at *pH* 7.9 in the presence of air. $c_{apo\text{-}MT1} = 1.83 \times 10^{-5}$ mol·l^{-1}. 1) apo-MT1; 2) adding 0.7 eq Cd^{2+}

Fig. 4. Being taken in the same steps and conditions, the CD spectra of apo-MT2 ($c = 0.68 \times 10^{-5}$ mol·l^{-1}) with the addition of 7 eq Cd^{2+} were obtained at *pH* 1.85, 2.72, 3.29, 4.31 (Fig. 5).

Result and discussion

Native MT polypeptide chain comprises of 20 cysteines out of a total of some 61 amino acid residues, thus being a sulfhydryl-rich protein. The study of two dimension NMR reveals that Cd^{2+} ion is coordinated tetrahedrally by thio-

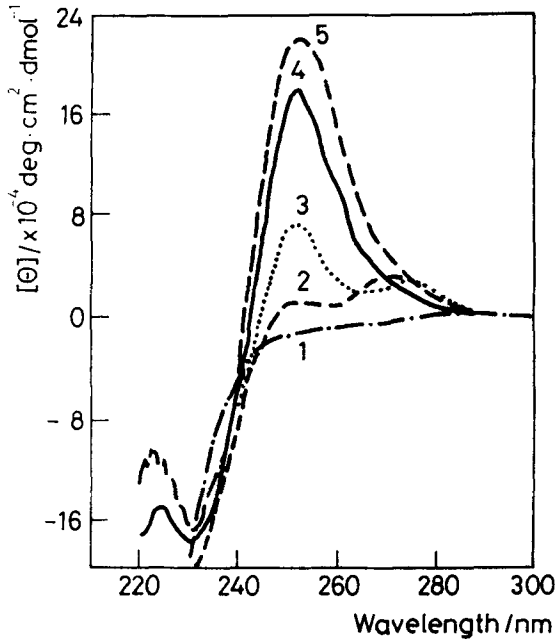

Fig. 4 The *pH* dependency of the CD spectra of apo-MT1 adding 7 eq Cd^{2+}, $c_{apo\text{-}MT1} = 1.07\times10^{-5}$ mol·l^{-1}. 1) *pH* 2.01; 2) *pH* 2.73; 3) *pH* 2.85; 4) *pH* 3.32; 5) *pH* 4.12

late groups in metal thiolate cluster structure [10] that is composed of α-(four Cd^{2+} ion are coordinated by 11 sulfhydryls) and β-(three Cd^{2+} ion are coordinated by 9 sulfhydryls) domains of Cd_7-MT in solution, in agreement with the result of X-ray diffraction crystallographic analysis [11]. The static structure of metallothionein crystal provides the base of recognizing the relations of functions and structures of metallothionein. It is also important to study the behaviours of MT releasing metals and apo-MT binding metals in solution, because it may be concerned with the change of the dynamic structure of metallothionein in organism when MT acts in the role of biological functions. Since MT molecular steric structure depends on the folding of polypeptide chain resulted from the coordination of sulfhydryls with metal ions, factor which can affect the stability of sulfhydryls and its complexation with metal ions will affect the conformation of MT. Among many factors, the type and quantity of metal ions and the *pH* value of solution are the most important two.

The effects of pH value on stability of apo-MTs' sulfhydryls

Experiment data are shown in Table 1. The remnant sufhydryls of apo-MT1 measured are 99–100% at *pH* 4.71 and 10% at *pH* 7.9. Present experiments

demonstrate that number of sulfhydryls has not changed obviously if apo-MT is placed in 4°C air at *pH* 2 for 24 h; sulfhydryls of apo-MTs can exist stably for one day when *pH* value is lower than 5, but they will decrease remarkably if *pH* value is higher than 5.

The CD spectra of apo-MT1 binding Cd^{2+} ion in successive steps

A shoulder appears at 225 nm on the CD spectrum 1 of apo-MT1 (Fig. 1) with a big negative peak at 205 nm (not shown in this paper) at *pH* 4.71, which is same as that of apo-MT1 at *pH* 1.92 [7] reflecting the characteristic of the mainly disordered structure of polypeptide chain of apo-MT [12]. There are small peaks or inflexion points at 256, 234 and 225 nm on CD band as adding 2 eq Cd^{2+} to apo-MT1 solution. The CD spectra of apo-MT1 binding 7 eq Cd^{2+} at *pH* 4.71 with the ellipticity peaks at 257 nm(+), 238 nm(−), 226 nm(+) are the same as those of native MT Cd-induced at *pH* 4.44 manifesting themselves the characteristic conformation of α- and β-domains formed by Cd^{2+} thiolate coordination [7]. The positions of peaks are shown more clearly on a differential CD spectra (Fig. 2). The experiments indicate that the CD spectra as adding 2 eq Cd^{2+} ion into apo-MT1 have the same pattern of that of adding 7 eq Cd^{2+} ion except for the increasing of ellipticity amplitude with the increase of Cd^{2+}-to-protein ratio, which demonstrates the cooperation effect present in the formation of cadmium thiolate cluster.

In the presence of air, the CD spectra of apo-MT1 solution with Cd^{2+} ion at *pH* 7.9 are quite different from those at *pH* 4.71, and there is only one peak at 243 nm shown as Fig. 3. CD spectra do not change after adding 0.7 eq Cd^{2+} ion at *pH* 7.9, confirming the inference that apo-MT1 can bind at most 0.7 eq Cd^{2+} ion because only 10% of sulfhydryls remain unoxidized under this condition.

Different patterns of CD spectra of apo-MT1 binding Cd^{2+} ion in successive steps are correlated with different structures of complexes. In the presence of air, about only two sulfhydryls (10%) per apo-MT1 molecule in solution are unoxidized at *pH* 7.9 on average. In spite of the positions of remnant sulfhydryls indefinite, it is sure that the normal conformation of MT can not be constructed with their coordination to Cd^{2+}, whereas an intramolecular (if number of remanent sulfhydryls ≥4) or intermolecular (Cd^{2+} ions are binded randomly) mononuclear Cd-thiolate coordination may be formed. The peak at 243 nm(+) arose from this structure in present paper unwittingly confirms the H. Willner's conclusion [13]. Their experiments were performed with exclusion of air, CD spectra of rabbit liver apo-MT2 with incremental addition of Cd(II) were recorded at *pH* 8.4. They observed that there was only one peak at 243 nm(+) when the first 3 equiv Cd(II) were added, identified as arising from mononuclear tetrahedral Cd-tetrathiolate complexes, whereas the peaks at 260 nm(+),

240 nm(−), 224 nm(+) emerge on CD spectra after addition of the remaining 4 eq Cd(II) identified as arising from doubly coordinated bridging thiolate ligands in the Cd-thiolate clusters. So they suggested that a transformation from mononuclear tetrahedral Cd-tetrathiolate complexes to Cd-thiolate clusters containing bridging thiolate ligands took place in the process of apo-MT2 binding Cd(II) ion in successive steps. In contrast, the apo-MT1 is different from apo-MT2 that the formation of Cd-thiolate cluster containing bridging thiolate ligands is completed in one step when Cd^{2+} ion are bound to apo-MT1, however the transformation from mononuclear complex to multinuclear complex did not appear as shown in Fig. 1. This fact implicates that there are differences in binding sites and pathway to bind Cd^{2+} ion between apo-MT1 and apo-MT2, not reported previously.

In addition, the present results indicate that only under certain conditions a normal cluster structure could be formed if polypeptide chain folding was formed due to the coordination of sulfhydryls to metal ion in sufficient sulfhydryl to metal ion ratio. In the presence of an oxidant or free radical, MT's native conformation can not be formed by reconstitution of apo-MT with metal ion at high *pH* value, resulting in a disorder of metal metabolism with participation of MT/apo-MT molecular pair.

pH dependency of the CD spectra of reconstituted MTs by apo-MTs with 7 eq Cd^{2+}

From Fig. 4, a shoulder appears in line 1 even apo-MT solution with 7 eq Cd^{2+} ion at *pH* 2 that is similar to the CD spectrum of apo-MT shown in Fig. 1. It demonstrates that sulfhydryls can not coordinate with Cd^{2+} ion, and normal conformation of MT can not be formed at *pH* 2 although there are sufficient Cd^{2+} ion in the system. It was reported that Cd-induced native rabbit liver MT began to release Cd^{2+} ion at *pH* 3.8, and Cd^{2+} ion were lost completely at *pH* 2.8 [7], so that apo-MT can not complex with Cd^{2+} ion at *pH* 2. However, apo-MT can complex with Hg^{2+} ion at *pH* 2 and Hg_7-MT does not release Hg^{2+} ion above *pH* 0.84 [8]. This difference between Cd(II) and Hg(II) in their ability of complexing with apo-MT indicates that their affinities for thiolate ligands are different. Metal with strong affinity can complex with apo-MT at a low *pH* value and substitute metal with weak affinity from MT directly. The stability of Zn-thiolate cluster of native MT is weaker than those of Cd, Hg, which is the base of detoxication of MT for heavy metals [4].

The changes of CD spectra with increasing *pH* indicate that apo-MT1 comes to complex with Cd^{2+} ion. It is remarkable that two small peaks appear at 252 and 273 nm at *pH* 2.73 and 2.58, while the peak at 273 nm decreases until disappearance with increasing *pH*. A typical DC spectrum of Cd_7-MT1 with metal-cluster structure displays peaks at 254 nm(+), 234 nm(−) and 220 nm(+) at

pH 3.32, indicating that the conformation of reconstituted MT1 with Cd^{2+} ion is the same as that of native MT1 [7]. It was discovered from the study of changes of native MT1 conformation with *pH* that the peak at 256 nm split into two small peaks [7] too, with a small peak appearing near 271 nm, that is consistent with the result of this paper.

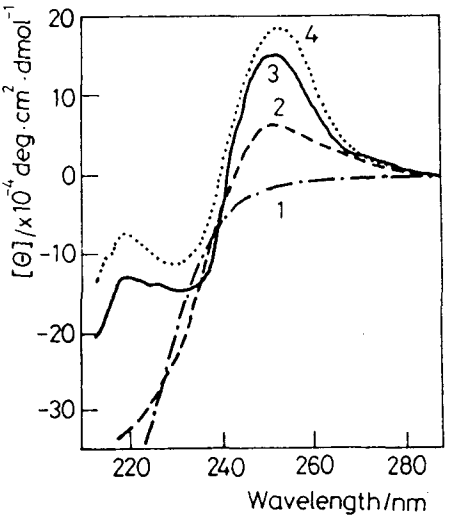

Fig. 5 *pH* dependency of the CD spectra of apo-MT2 adding 7 eq Cd^{2+}. 1) *pH* 1.85; 2) *pH* 2.72; 3) *pH* 3.29; 4) 4.31, $c_{apo\text{-}MT2}=0.68\times10^{-5}$ mol·l^{-1}

The change of CD spectra of apo-MT2 + 7 eq Cd^{2+} system with *pH* is quite different from that of apo-MT1. From Fig. 5, the position of peak at 254 nm does not change with *pH* from 1.85 to 4.31, and the two small peaks that appear on CD spectra of MT1 do not appear. The CD spectra of reconstituted MT2 with peaks at 254 nm(+), 234 nm(−), 220 nm(+) are in agreement with those of MT1, that can be explained as they have the same metal-cluster structure.

The above results reveal that conformation of MTs reconstituted by apo-MT1 and apo-MT2 with 7 eq Cd^{2+} is the same as that of native MTs, but the pathway of binding Cd^{2+} ion of the two isoforms may be different. There are a few differences between rabbit liver MT1 and MT2 in amino acids composition and sequence, that MT2 is devoid of one arginine and one threonine residue than MT1, but containing one extra serine and one lysine residue [15]. It is supposed that $>C=NH^{2+}$ double-bond of arginine residue (20th amino acid residue on apo-MT1 polypeptide chain) may complex with metal ion with a π-bond at first, and participate in the formation of β-domain subsequently, related to the appearance of the two peaks at 252 nm and 273 nm on CD spectra of MT1 at *pH* 2.73, 2.85 and 2.94. Authors suggest that coordination of sulfhydryls plays

a key role when apo-MT1 and apo-MT2 bind Cd^{2+} ion, and the π-bond complexation with the participation of arginine residue of apo-MT1 strengthens the stability of MT1. It can be predicted that, for one kind of metal, the ability of binding metals of apo-MT1 is stronger than that of apo-MT2, whereas, for MTs containing same metal, the ability of releasing metals of MT1 is weaker than MT2. There are some examples such as the ability of binding Hg^{2+} ion of apo-MT1 is stronger than apo-MT2 [8], and the order of abilities of removing hydroxy free radical and anti-radioactivities of MT isoforms in Zn-MT2>Zn-MT1>Cd-MT2>Cd-MT1 [3]. Further studies are necessary to provide direct evidences of the π-bond complexation and to reveal the biological significance of differences between MT1 and MT2. A useful clue is provided in this paper to explore the mechanism.

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Zusammenfassung — Der Zirkulardichroismus wurde zur Untersuchung der Komplexierung zweier Isoformen von Leber apo-Metallothioneinen beim Hasen (apo-MT1 und apo-MT2) mit dem Cd^{2+}-Ion und des Einflusses des Cd^{2+}-Ions auf die Konformation von neukonstituierten MT. Die Stabilität der Sulfhydryle von apo-MT wurde bei Raumtemperatur in Gegenwart von Luft untersucht. Die Neukonstitutionen von apo-MT1 mit Cd^{2+}-Ion wurde bei pH 4.71 (stabiler Zustand) und *pH* 7.9 (90 % der Sulfhydryle oxidiert) durchgeführt. Man fand, daß die charakteristische CD-Bande bei 257 nm(+), 238 nm(-) und 226 nm(+) von neukonstituiertem MT mit Cd^{2+}-Ion die gleiche war wie für natives MT bei *pH* 4.71, und lediglich ein Peak bei 243 nm(+) trat im CD-Spektrum bei *pH* 7.9 auf, der von mononuklearen Komplexen mit vier separierten Thiolatliganden pro Cd^{2+}-Ion stammt.
Das CD-Spektrum des Systemes apo-MT + 7 Äquiv.Cd^{2+} wurde bei verschiedenen *pH*-Werten vermessen. Man fand, daß der Peak bei 256 nm der Bindung von apo-MT1 an das Cd^{2+}-Ion im

Bereich von *pH* 2.42 und 3.02 in zwei kleinere Peaks gespaltet wird und bei *pH* 3.32 wieder zu einem Peak wird, während die Gestalt der CD-Peaks der Bindung von apo-MT2 an das Cd^{2+}-Ion *pH*-unabhängig ist, was darauf hinweist, daß die Bindungsstellen und -wege von apo-MT1 zur Bindung des Cd^{2+}-Ions sich von denen von apo-MT2 unterscheiden. Ein möglicher Mechanismus wird vorgeschlagen.