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Cadmium binding studies to the earthworm *Lumbricus rubellus* metallothionein by electrospray mass spectrometry and circular dichroism spectroscopy

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Abstract

The earthworm *Lumbricus rubellus* has been found to inhabit cadmium-rich soils and accumulate cadmium within its tissues. Two metallothionein (MT) isoforms (1 and 2) have been identified and cloned from *L. rubellus*. In this study, we address the metalation status, metal coordination, and structure of recombinant MT-2 from *L. rubellus* using electrospray ionization mass spectrometry (ESI-MS), UV absorption, and circular dichroism (CD) spectroscopy. This is the first study to show the detailed mass and CD spectral properties for the important cadmium-containing earthworm MT. We report that the 20-cysteine *L. rubellus* MT-2 binds seven Cd^{2+} ions. UV absorption and CD spectroscopy and ESI-MS pH titrations show a distinct biphasic demetalation reaction, which we propose results from the presence of two metal–thiolate binding domains. We propose stoichiometries of Cd_3Cys_9 and Cd_4Cys_{11} based on the presence of 20 cysteines split into two isolated regions of the sequence with 11 cysteines in the N-terminal and 9 cysteines in the C-terminal. The CD spectrum reported is distinctly different from any other metallothionein known suggesting quite different binding site structure for the peptide.

Keywords: Metallothionein; Earthworm *Lumbricus rubellus*; Cadmium; Electrospray ionization mass spectrometry; Circular dichroism spectroscopy; UV absorption spectroscopy; Biomarker; Metal coordination; Protein structure; Metalation

Earthworms have been found to survive in heavy metal-contaminated mine soils [1–3] with levels exceeding 34 mg arsenic (As) g^{-1} dry soil weight [2,4], 350 μg cadmium (Cd) g^{-1} dry soil weight, and 3 mg copper (Cu) g^{-1} dry soil weight [5]. Due to the intimate nature in which earthworms interact with soil including dermal contact and ingestion, earthworms are likely to accumulate toxins such as metals and metalloids [6]. Indeed, cadmium, arsenic, and copper are accumulated in the earthworms inhabiting these metal-contaminated mine sites [1,5,7,8]. This ability to live in the presence of toxic metals has sparked interest in their possible application as biomarkers for metal and metalloid accumulation. However, use of earthworms as biomarkers requires the quantification of the behavioral, physiological, and gene expression changes that occur upon exposure to these toxins [9]. Moreover, detailed knowledge of the resulting protein induction, the capacity for metal and metalloid binding as well as the elucidation of the underlying mechanisms of the interactions is necessary to enable the prediction of the toxicology of these biomarkers.

In earthworms, Cd bioaccumulates in distinct organelles by a bioconcentration factor of 2.45 [10]. Studies using electron probe X-ray microanalysis (EPXMA) and X-ray absorption spectroscopy (XAS) on tissues obtained from earthworms resident in former mine sites have shown Cd to be coordinated to sulfur ligands and (based on the Cd–S bond distance) it was proposed that metallothionein (MT) was the chelator [10]. MT in earthworms is induced

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by Cd [11] within 3 days of dermal exposure [12]. Immunostaining has shown that MT is concentrated in the chloragogenous tissue surrounding the alimentary canal and possibly in acidic subcellular structures bearing a resemblance to lysosomes, and thus, more recently referred to as cadmosomes [10,13].

Metallothionein is a ubiquitous metal-binding protein found in plants, invertebrates including earthworms, and mammals, as well as many other organisms. Unique to MT is its small size (approximately 60–80 amino acids) and high cysteine but low aromatic amino acid content. Studies on MT have shown that this small protein can bind a range of metals including, but not limited to, Cd^{2+}, Zn^{2+}, Cu^{+}, Bi^{3+}, Hg^{2+}, and As^{3+}. Mammalian MTs bind seven Cd^{2+} in two metal-binding domains, forming Cd_3Cys_9 (β) and Cd_4Cys_{11} (α) metal-cysteine clusters at the N- and C-terminal, respectively [14–17].

Mass spectrometry (MS) studies of metallothionein have shown that the nematode *Caenorhabditis elegans* MT-2 binds Cd with a stoichiometry of up to Cd_6Cys_{18} [18] yet there have been no MS studies on the important earthworm MTs. Sturzenbaum et al. [19,20] have identified and cloned two MT isoforms from *Lumbricus rubellus*, which contain 20 cysteines and one of the recombinant isoforms is used in this study. *L. rubellus* MT (wMT) isoforms 1 and 2 were reported to bind approximately 6 Cd^{2+} based on a basic Cd^{2+} titration by UV spectroscopy [19]. This paper focuses on recombinant (r) wMT isoform 2, abbreviated rwMT-2, which is the Cd-inducible earthworm MT isoform [19].

There have been no reports of structural studies on earthworm MTs to date. CD spectroscopy studies can be used as an initial probe into a protein's structure and in this paper we directly address this lack of structural detail by providing the first structural information for this important class of proteins. Mass spectrometry, UV absorption, and CD spectroscopy studies carried out in this paper address the exact metalation status, coordination and structure of rwMT-2 with respect to cadmium, the first time such data have been reported for any earthworm MT.

Materials and methods

Chemicals. $CdSO_4$ (Fisher Scientific). All chemicals used in this study were of the highest-grade purity from commercial sources. Hi Trap SP ion exchange columns and G-25 Sephadex (Amersham Biosciences) were used for protein purification.

Protein preparation. The recombinant *L. rubellus* metallothionein (rwMT-2) protein used in this study was based on the 77-residue sequence: MADAFNTQCC GNKTCPREGS TCACSKCRCP KDDCAPNCKK LCCADAQCGN ASCSCGAACK CAAGSCASGC KKGCCGD. There are 20 cysteine residues present and no disulfide bonds in this sequence. In addition to the sequence from the rwMT-2, the expression system includes the amino acid residues of the stabilizing S-peptide tag (MKETAAAKFERQHMDSPDLGTLVPRGS) on the N-terminus of the fragment [19,20]. Recombinant rwMT-2 was expressed in BL21(DE3) *Escherichia coli* cells that were transformed using a pET29a plasmid. Further protein purification and desalting was performed by elution through a Sephadex G-25 column using 25 mM ammonium formate, pH 7.4 (Fisher). Elution was monitored by UV–Vis absorption between 200 and 300 nm. Fractions that contain rwMT-2 were collected and the purity was assessed by electrospray ionization mass spectrometry (ESI-MS). The protein was kept in its reduced state through careful deoxygenation of the sample by evacuation and saturation with argon gas such that the protein was contained within a sealed inert environment. Demetalation was by acidification with solutions of 0.12, 2.4, and 6 M HCl.

ESI-MS procedures. All data were collected using a Micromass LCT mass spectrometer in the positive ion mode. The mass spectrometer was operated using the parameters: 3000.0 V capillary, 42.0 V sample cone, 22.0 V extraction cone, acquisition scan time of 4 s, and interscan delay time of 0.4 s. The ESI-MS data were processed and deconvoluted using the Max Ent I software (Micromass). The instrument was calibrated using a mixed NaI and CsI solution.

Spectroscropic measurements. The UV–Vis absorption spectroscopy was performed on a Cary 500 UV–Vis spectrophotometer in the absorbance mode for the wavelengths 300–200 nm. A Jasco J810 was used to perform the circular dichroism (CD) spectroscopy between 200 and 300 nm with a bandwidth of 1 nm.

Results and discussion

Mass spectra of rwMT-2

We show the ESI-MS data for the apo-, Cd_4-, and Cd_7-rwMT-2 species in Fig. 1; the charge state is shown on the left-hand side and the calculated parent species is shown on the right-hand side. At pH 6.8, the dominant charge state present is +7, while at pH 2.3, the dominant charge state is +9. There are 14 basic amino acids available for protonation at low pH as shown in the sequence. For both experimentally chosen pH values, the dominant charge states are much less than the maximum of 14 basic amino acids shown in the sequence. This suggests that the protein adopts a closed structure that prevents complete solvent access at both neutral and low pH. Further, it can be seen from the small change in the dominant charge state that, following demetalation, the protein unfolds allowing for greater solvent access. However, significantly, the protein does not completely unfold so that the charge state maximum only increases to +9. This suggests the presence of a residual structure even for the metal free, apo-rwMT-2, a property reported previously [21–24].

Based on the primary sequence shown in Fig. 1, the expected masses for metal free, Cd_4- and Cd_7-protein are 10,609.3, 11,048.0, and 11,376.2 Da, respectively. The measured masses match reasonably well with the expected mass. Whilst Sturzenbaum et al. [18] reported that rwMT-2 binds approximately six Cd^{2+} ions, our more accurate ESI-MS data determined that rwMT-2 binds 7 Cd^{2+} ions. Though XAFS have provided bond lengths, the exact metalation status for earthworm MT in those studies was not known. It is well established that mammalian MTs, which also have 20 cysteines, bind 7 Cd^{2+} ions in well-defined clusters [14–17].

pH studies can provide information about possible cluster and domain structures, by indicating the sequence of demetalation as a function of increasing proton concentrations, where the protons compete for the Cd^{2+} sites on the sulfurs. The lower the binding constant, the higher the pH

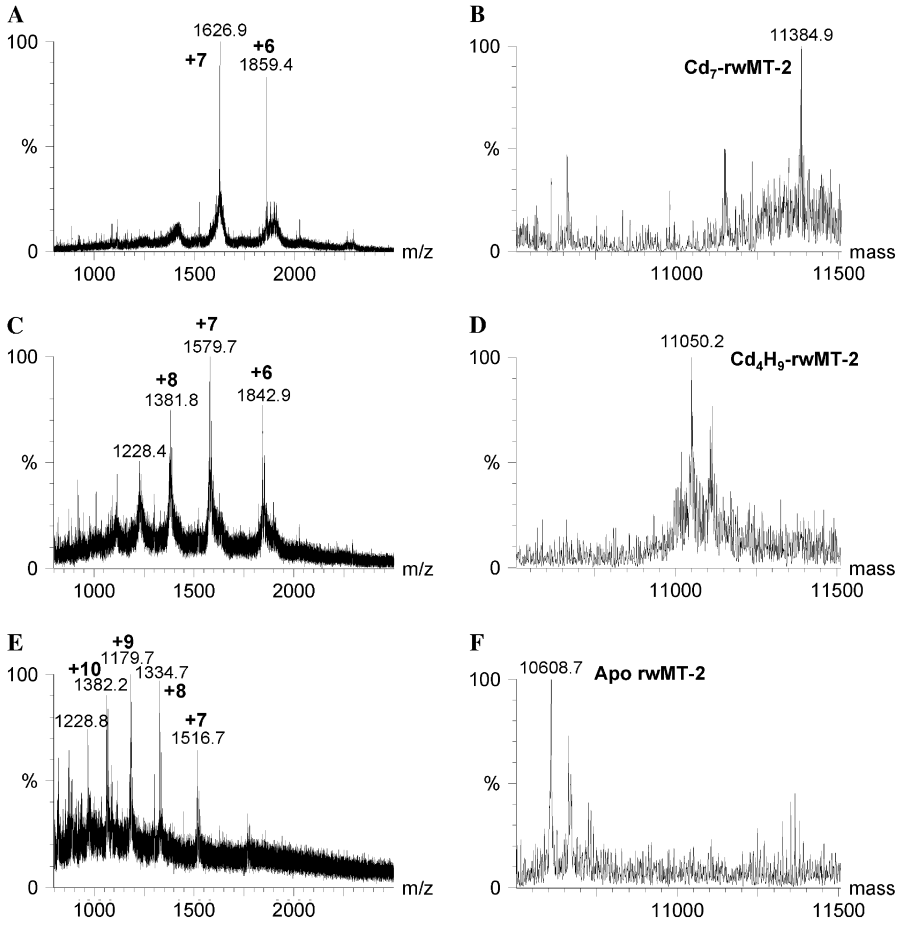

Fig. 1. The observed charge states (A, C, and E) and the reconstructed spectra (B, D, and F) for Cd_7- (A,B), Cd_4- (C,D), and apo-rwMT-2 (E,F) using ESI-MS. The measured mass for Cd_7-rwMT-2 at pH 6.80 is 11384.9 Da (B), for Cd_4-rwMT-2 at pH 3.46 is 11050.2 Da (D), and for apo-rwMT-2 at pH 2.30 is 10608.7 Da (F). All samples were buffered in 25 mM ammonium formate.

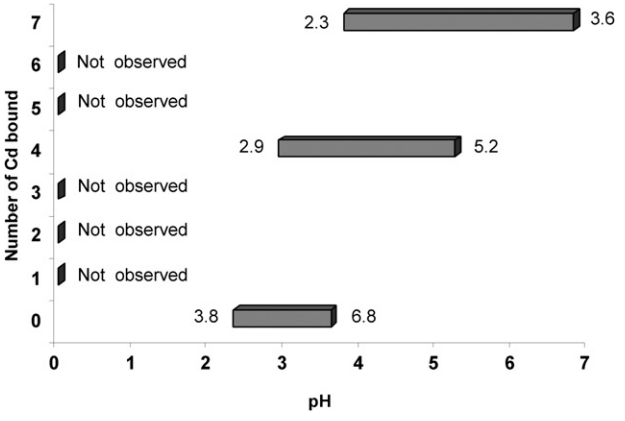

Fig. 2. pH dependence of Cd^{2+} speciation and stoichiometry in rwMT-2 using ESI-MS. Bars represent the pH range for each species as detected by the ESI-MS. The relative abundances of each species change according to the pH.

that the cysteines begin to be protonated and the metal released. The sequence of rwMT-2 shows 20 cysteines, the connectivity between each cysteine residue and the bound Cd^{2+} is not currently known. Based on the Cd stoichiometry and the preferred tetrahedral coordination geometry for Cd, which requires four ligands, we propose that there are bridging cysteinyl sulfurs present and are used to form adamantane-like clusters only in the one domain. From the pH titration in Fig. 2, we can see that 3 Cd^{2+} are released first followed by the remaining 4 Cd^{2+} at a lower pH.

UV absorption and CD spectra

UV absorption and CD spectra confirm that the Cd^{2+} ions are bound to cysteinyl sulfurs, as the characteristic ligand to metal charge transfer band for Cd–cysteine complexes is observed at 254 nm. Protonation of the cysteinyl sulfurs results in the release of Cd^{2+} ions and the reduction in the intensity of the 254 nm band [17]. Therefore, the UV absorption spectrum of the cadmium-containing rwMT-2 is quite similar to the spectra of other Cd-MTs [25].

The best known CD spectrum of MT is that of the mammalian Cd_7-MT, particularly, rabbit liver Cd-MT-2A [25]. Key features that are seen in the CD spectra of the mammalian MTs (for example [25]) are, in decreasing wavelength order, a $(+,-,+,-)$ sequence starting at 260 nm

(+), 240 nm (−), 225 nm (+), and 210 nm (−), where the maximum at 260 nm does not lie under any specific absorption band, whereas the cross-over at 250 nm does. These spectral features have been associated with CD exciton coupling effects of the 4 Cd^{2+} atoms in the α domain because at lower symmetry (with 1, 2 or 3 Cd^{2+}) the CD maximum is measured at 250 nm [25]. The CD spectrum shown of the cadmium-containing earthworm MT is distinctly different from any previously published data. The spectral features in this region exhibit a (−,+,−) sequence starting at 263 nm (−), 243 nm (+), and 225 nm (−). As CD spectroscopy is a reflection of the peptide wrapping, and the spectral bands observed are cysteine to Cd^{2+} charge transfer in origin, we conclude that the earthworm and mammalian MT peptides wrap around cadmium in a completely different orientation. However the Cd cluster core, that is the Cd–thiolate structure, may still be similar to mammalian MTs as other studies involving crab, lobster, and retro-α MT (where the sequence of the protein is reversed) have shown how well conserved the Cd–S_{cys} cluster core is even when the peptide wrapping is dissimilar [26–29].

Measurements of the UV absorption and CD spectra during acidification of rwMT-2, Figs. 3 and 4, show a two-step process in which there is a distinct reduction in intensity at approximately pH 3.5, suggesting that the demetalation takes place in two phases. This correlates well with the MS results that show a biphasic demetalation. Fig. 3 shows that for each of the two steps in the demetalation, there is a loss of approximately 50% in absorbance.

Although the number of cadmiums bound (7) is analogous to the Cd-content in mammalian MTs [30,31], this does not imply that earthworm and mammalian MT, both of which contain 20 cysteines, may adopt identical binding motifs. Indeed, the 11-cys segment (the α domain in mammalian MT) is at the N-terminus and the 9-cys segment (the β domain in mammalian MT) is at the C-terminus, which is the reverse of mammalian MT, but coincides with

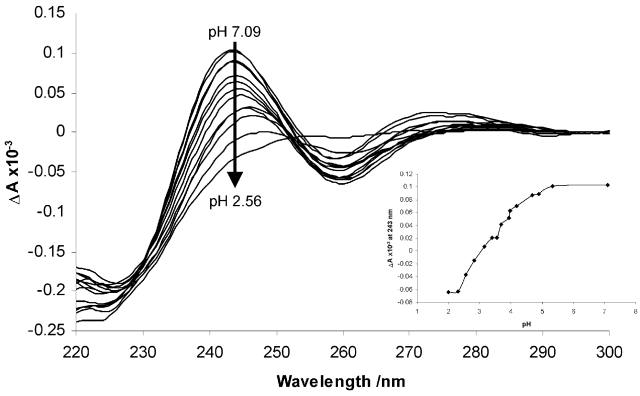

Fig. 4. CD spectra measured during the acidification of rwMT-2 with HCl. Inset shows the change in CD intensity as a function of pH at 243 nm.

other worm MTs [19,32]. In unpublished work (J. Chan, P. Kille, I. Watt, Z. Huang, and M.J. Stillman) the reversed human αβ-rMT-1 was shown to adopt the same motif as the human βα-rMT-1, which suggests that the location of the individual segments of peptide that form the two clustered binding sites is not as significant as it might seem in determining the stoichiometry and structure of the domains. Pan et al. [26] constructed a human retro-α domain protein showing that the Cd cluster core appeared to be similar to the normal α domain, though the actually wrapping may be different. However, it is clear that Cd-NMR studies are required to provide the experimental evidence for the exact Cd-cys connectivities in this novel worm MT.

Conclusions

The studies reported here provide strong evidence that rwMT-2 from *L. rubellus* coordinates seven Cd^{2+} ions in two domains with stoichiometries of Cd_3Cys_9 and Cd_4Cys_{11}. Cluster formation of earthworm MT occurs,

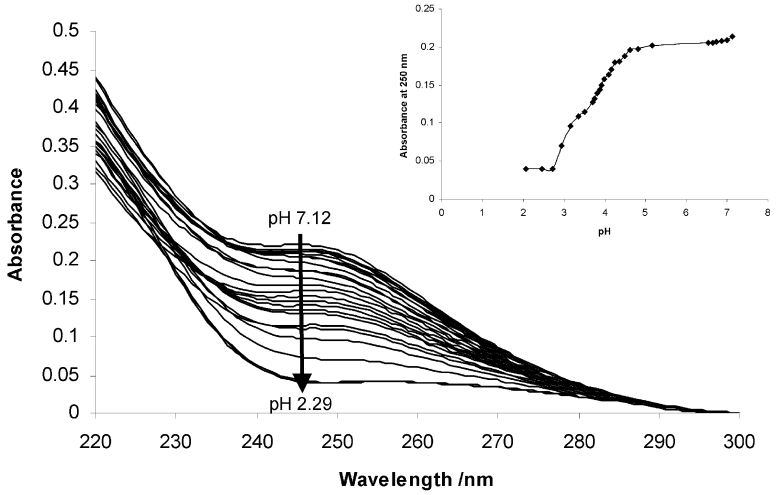

Fig. 3. UV absorption spectra measured for the acidification of rwMT-2 with HCl. Inset shows the absorbance of the cysteinyl sulfur-to-cadmium charge transfer (LMCT) band at 250 nm as a function of pH in which there is a distinct step at pH 3.5.

but the CD spectrum shows that the overall protein structure may be different to its 20-cysteine mammalian counterpart.

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