Metal Ion Trafficking in Earthworms

IDENTIFICATION OF A CADMIUM-SPECIFIC METALLOTHIONEIN*

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Exposure to cadmium poses a considerable risk to human health and environmental safety. Earthworms reside in the most contaminated sites on earth, displaying a phenomenal tolerance to toxic heavy metals. They exhibit a distinct metabolic pathway that allows the bio-accumulation of cadmium to yield body burdens in excess of 1/1000th of total dry body weight, a most impressive figure by any standard. However, the precise molecular mechanism underlying this phenomenon remains to be unraveled. This study meets this challenge by fully characterizing the major metal-binding protein in earthworms, namely the two isoforms of metallothionein. Chemical analysis of recombinant protein showed that although both isoforms bind equimolar amounts of cadmium (6 mol), wMT-2 is more stable during proton competition. Furthermore, isoform-specific transcript analysis demonstrated that only wMT-2 is responsive to cadmium in a dose and temporal manner. The specific sequestration of cadmium to wMT-2 protein was confirmed in situ using polyclonal antisera. The latter also provided the means for mapping the cellular and intracellular distribution of metallothionein, thus yielding a holistic insight into its involvement in cadmium transit during absorption, storage, and excretion. The structure-function relationship of wMT-2 and its role in cadmium detoxification through sequestration and compartmentalization is discussed.

Earthworms are central to soil quality and fertility. The first authoritative account of the central role of the earthworm in soil formation, quality, and fertility dates back to Charles Darwin well over 100 years ago (1). More recent studies have shown this oligochaete annelid exhibiting an exceptional tolerance to cadmium, residing in soils with concentrations exceeding 600 μg of cadmium/g dry weight (2). In addition to surviving this toxicological challenge, they bio-accumulate this metal ion to a body burden in excess of 1 mg/g dry weight (2, 3). This accumulation of cadmium is in stark contrast to the exclusion observed with other metal ions, for example, copper (4). However, even where ions with closely related chemistry, such as cadmium and zinc, are elevated within the same environment, the compartmentalization, and therefore the metabolic pathway, remains distinct (5). Therefore, the earthworm must have a highly developed and specific trafficking pathway for this toxic metal ion. Ultrastructural mapping has shown that the majority of the cadmium is sequestered as a thiol-based inorganic complex within intracellular vesicles in the earthworm’s primitive liver-like tissue, the chloragog (5).

The co-localization of cadmium and sulfur within a distinct intracellular organelle suggested the involvement of a sulfur-rich protein. Metallothioneins (MTs) are cysteine-rich cationic proteins that have been linked to cadmium detoxification in a wide range of phylogenetic orders (e.g. humans (6), fish (7), Caenorhabditis elegans (8), springtails (9), and others). However, until recently (2) no molecular information has been forthcoming regarding metallothionein from annelid species. These authors (2) describe the cloning of two MT isoforms that encode the major cadmium-binding protein in earthworms and demonstrated a potential transcriptional up-regulation of 3 orders of magnitude when resident on a cadmium-contaminated soil. Elegant mass spectral analysis of MT protein fragments supported these observations (10). Three problems prevented detailed mechanistic investigations. Firstly, the protein isolated from earthworms was insufficient to initiate a full chemical analysis. Secondly, the small amount of protein obtained consisted of a protein pool containing a mixture of isoforms. Thirdly, gene quantification was isoform-nonspecific and thus only provided a measure of total MT transcripts. These constraints have precluded investigations into possible isoform-specific functions, as observed previously in a terrestrial mollusc (11).

In the present study we overcame these limitations through the production of recombinant proteins of both isoforms, thereby providing sufficient material to enable the initiation of full chemical characterizations as well as the production of isoform-specific MT antisera. The latter provided the means for mapping the cellular and intracellular localization of MT by light and electron microscopy, thus yielding an important in situ insight into the functions of MT in metal sequestration and excretion within defined cell types. Combining these data with a new generation of isoform-specific transcript analyses, we were able to generate a holistic understanding of the role(s) of MT in cadmium toxicology at a molecular, cellular, and whole systems level in a higher eukaryote, which is of considerable applied significance in environmental sciences.

EXPERIMENTAL PROCEDURES

Isoform-specific Quantitative PCR—Individual earthworms (Lumbricus rubellus) were maintained on Kettering loam soil (Broughton Loam Ltd., United Kingdom) under constant laboratory conditions in the

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AJ005822 and AJ005823

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1 The abbreviations used are: MT, metallothioneins; PCR, polymerase chain reaction; XRMA, x-ray microanalysis; LM, light microscopy; EM, electron microscopy; FPLC, fast protein liquid chromatography.
absence of, or supplemented with, sublethal concentrations of CdCl_2 (0, 30, or 300 μg/g of dried soil) over a standard time course (1, 3, 7, and 21 days). Total RNA was isolated with TRI reagent (Sigma) and reverse transcribed using standard protocols. MT quantifications were subsequently performed on an ABI PRISM® 7700 (PerkinElmer Life Sciences) using probes and primers designed using the program Primer Express® (PerkinElmer Life Sciences). All amplifications were isoform-specific and devoid of detectable cross-reactivity. Each data point represents (actin-normalized) triplicate MT measurements performed on each of four individual earthworms.

**Amplification and Cloning of MT Constructs**—wMT-1 and wMT-2 were amplified from cDNA cloned into isoform-specific primers containing 5’ SaI and NcoI restriction site extensions. The PCR products were allowed to grow to an overnight culture and ligated using 2 units of T4 ligase and 1X ligation buffer (Promega) and transformed into DH5α containing competent cells (Life Technologies, Inc.). DNA was prepared (Miniprep, Promega) from positive colonies, identified by PCR screening, and stored at -80°C. Furthermore, sequence identity was confirmed by PCR-based Thermo Sequenase fluorescent-labeled primer sequencing.

**Expression and Purification of S-Tag-MT Fusion Protein**—The cells were allowed to grow to an OD_600 of 0.6 followed by the induction of protein expression through the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (Promega). Cadmium concentration was increased by 50 μM every hour to stabilize the expressed MT and reduce essential metal scavenging. After 5 h post induction, cells were harvested by centrifugation and lysed using a French pressure cell press (Aminco, Maryland). The cytosolic fraction was obtained by centrifugation at 10,000 x g for 60 min and concurrently heated for 10 min at 100°C. Precipitated proteins were removed by centrifugation. The supernatant was applied to a 5-mL SP Hi-trap Sepharose ion exchange column (Amersham Pharmacia Biotech) and equilibrated with 10 mM Tris-HCl, pH 7.4. The recombinant protein was eluted over a gradient of 0–200 mM NaCl using an FPLC system (Amersham Pharmacia Biotech). Cadmium levels were measured in each 5-ml elution fraction by atomic absorption spectrophotometry at 228.5 nm using a Varian SpectrAA-100 (Varian Instruments). In addition, the Cd-S thiol bond-specific absorbance (A_254) was measured using a Hewlett Packard diode array spectrophotometer. Cadmium-containing fractions were pooled and applied to a G-50 Sephadex column (20-ml void volume) eluting in MilliQ water. The cadmium-containing pool was used for amino acid analysis (Amersham Pharmacia Biotech) and pH titrations. Aporoprotein was prepared by exposure to anaerobic 0.1 M HCl as described previously (12) and separated from free cadmium by G-25 size-exclusion Sephadex chromatography. The concentration of apoprotein was determined by amino acid analysis (7), and the reduction state of cysteine residues was assessed in a 2,2′-dithiodiis(5-nitropyridine) assay (13). Proteins were resolved by SDS-polyacrylamide gel electrophoresis (14). Reconstitution of the apoprotein with cadmium was performed essentially as described in Ref. 15, and analysis of protein stability by pH titration was performed as published previously (16).

**Immunoperoxidase Localization of wMT by LM**—Whole worms were fixed in 10% formalin saline for 24 h and then processed for postembedding histochemistry. Fixed tissues were dehydrated in a graded alcohol series and embedded in paraffin wax. Sections (nominally 7 μm) were cut using a steel knife in a Bright microtome, mounted on Chance Gold Micro Slides, and dried overnight on a warming plate. Sections were examined after single immunolabeling using the indirect immunoperoxidase method essentially as described elsewhere (17).

**Electron Probe X-ray Microanalysis (XRMA) on Tissue Smears**—A small piece of fresh chloragogenous tissue was dissected from earthworms resident on cadmium-contaminated soil (Rudry, South Wales, UK, see (2)), smeared across a 3-mm 100 mesh titanium electron microscope grid, and allowed to dry air (18). Grids were examined in a JEOL JEM1210 transmission electron microscope operated at 80 kV and fitted with a LINK ATW Pentafet solid state x-ray detector (138 eV resolution) and a LINK ISIS multichannel analyzer (Oxford Analytical Instruments, UK). XRMA allowed cadmium-containing compartments to be located in the anhydrous preparations and chemically characterized in terms of associated elements with an atomic number > 40. The chemical information was obtained by quantitative XRMA on Thin Cryosections—Chloragogenous tissue from earthworms resident on cadmium-contaminated soil were fixed, cryoprotected, and nitrified. Thin sections were collected on copper grids for immunogold cytochemistry using the Tokuyasu method essentially as described by Webster (19). Sections were immediately adjacent to those used for MT immunolocalization were mounted on titanium grids. These were not exposed to uranyl acetate or any reagents involved in the immunodetection protocol. Sections on titanium grids were used for XRMA analysis of the MT-positive compartments in counterpart sections. Performing XRMA on chemically fixed material is a compromise (20) considered justified in the present application because cadmium, by definition, forms a stable complex with MT at circumneutral pH. Furthermore, XRMA could not be done on the Tokuyasu preparations because of the confounding presence of uranium signals in the spectra and neither can immunolocalization be performed on unfixed cryosections. Nevertheless, the conjugation of two otherwise incompatible techniques on adjacent sections provided complementary information on the subcellular distribution of cadmium and MT.

**RESULTS**

**Isoform-specific Quantitative PCR**—The encoded proteins of two earthworm *L. rubells* MT isoforms display an identity of 74.7% and a similarity of 91.1%. *B*. isoform-specific transcript analysis of wMT-1 (i) and wMT-2 (ii). Quantifications were performed on an ABI PRISM® 7700 and normalized with actin. Triplicate measurements were taken of four individual earthworms sampled from a temporal- and dose-exposure experiment. The main graphical output in panel B elicits the relationship between wMT-1 and wMT-2 when exposed to cadmium over a time and dose course/gradient. wMT-2 is the sole cadmium-responsive isoform.
and showed a consistent correlation with the spectrally quantified cDNA concentration used as template. However, both isoforms of this oligochaete annelid worm showed an elevated transcriptional response during the initial time point, which returned to basal levels thereafter. It is well established that MT induction is stimulated by stress (21), and indeed the handling and acclimatization required at the start of the experiment could conceivably induce a stress response. Therefore, to investigate whether there was a differential transcription response between the two isoforms, the ratio of wMT-2 over wMT-1 was calculated (Fig. 1B). This clearly indicated that there is specific dose and temporal induction of wMT-2 in response to cadmium.

Recombinant Expression—To facilitate an extensive characterization at the protein level, each isoform was inserted into the pET vector system and expressed as S tag fusion proteins. The success of the accumulation of functional metal-binding proteins within the Escherichia coli cell was assessed by correlating the intracellular levels of cadmium with the induction of the recombinant protein. In comparison with the control (E. coli transformed with the empty pET 29a vector), an exponential temporal rise in cytosolic cadmium was observed in cultures expressing either of the MT isoform fusion proteins (Fig. 2A). An equivalent increase was observed in a protein band with a molecular mass corresponding to the relevant S tag fusion protein (Fig. 2B). Cation exchange chromatography was applied to purify the recombinant proteins to homogeneity, which when separated by SDS-polyacrylamide gel electrophoresis, migrated slightly slower than the native (S tag devoid) protein previously isolated from worms sampled from a cadmium-contaminated mine site (Fig. 2C).

Metal Stoichiometry and Stability—MTs bind metals via metal-sulfur thiol bonds which, in the case of cadmium, have a specific absorption shoulder at a wavelength of 254 nm. Exploiting the fact that the formation of cadmium-thiol bonds and protein-sequestered metal increases proportionally until MT is fully saturated, it was possible to assess the metal-to-protein ratio by stepwise molar reconstitution of an apo protein. Initially, metal ions were removed under anaerobic conditions from the purified recombinant MT by acidification and subsequent gel filtration. Independent concentration determination was performed using both DTNP and amino acid analysis, thus confirming that the generated apo protein was fully demetalated and that the thiol groups were reduced in excess of 97%. A stoichiometric addition of cadmium facilitated the reconstitution of holo protein with a stepwise increase in $A_{254}$ up to 6 molar equivalents of cadmium for both isoforms after which an absorbance plateau was observed (Fig. 3, A and B). Controlled demetalization through gradual increase in $H^+$ concentration was used to determine the stability of the cadmium clusters. These titrations showed that wMT-2 is able to retain its metal ions over a far wider pH range than wMT-1. This phenomenon is best quantified by comparing $K_d$ values, namely the $H^+$ concentration in which half of the metal is dissociated from the molecule. Although the wMT-1 dissociates at a pH of 3.3, the $K_d$...
of wMT-2 is 2.8, a value very similar to recombinant human MT expressed with an identical fusion tag (Fig. 3C). It is intriguing that the two isoforms bind equimolar amounts of divalent metal ion; however, the relative stability of their cadmium cluster(s) is significantly different.

Immunohistochemistry by LM—Polyclonal antibodies were raised against both recombinant earthworm MTs. Positive staining (red) was detectable within chloragogenous tissue in earthworms exposed to cadmium using both antisera (A). Competitive blocking, i.e. preincubation of antisera raised against wMT-2 with wMT-1 antigen and vice versa resulted in a complete loss of wMT-1 staining whereas no visual loss in intensity was detected after pretreatment of wMT-2 antisera (B). Essentially complete elimination of the positive signal is achieved by blocking simultaneously with both antigens (C) and similarly, no MT-positive staining was observed in control earthworms native to clean soil (D).

Fig. 4. Immunoperoxidase histochemistry was performed using polyclonal antibodies raised against both recombinant earthworm MTs. Positive staining (red) was detectable within chloragogenous tissue in earthworms exposed to cadmium using both antisera (A). Competitive blocking, i.e. preincubation of antisera raised against wMT-2 with wMT-1 antigen and vice versa resulted in a complete loss of wMT-1 staining whereas no visual loss in intensity was detected after pretreatment of wMT-2 antisera (B). Essentially complete elimination of the positive signal is achieved by blocking simultaneously with both antigens (C) and similarly, no MT-positive staining was observed in control earthworms native to clean soil (D).

Immunohistochemistry by LM—Polyclonal antibodies were raised against both of the cadmium-loaded recombinant earthworm MT isoforms. The antisera generated showed negligible cross-reactivity to recombinant human MT as well as the S tag peptide. In contrast, a competitive enzyme-linked immunosorbent assay confirmed that the antibodies specifically identified both native and recombinant earthworm MTs (data not shown). Furthermore, MT-positive immunoperoxidase staining was identified using both antisera, as indicated by intense staining within chloragogenous tissue from earthworms native to a cadmium-contaminated location (Fig. 4A). Being polyclonal antibodies, they initially did not exhibit isoform specificity. However, antibody specificity could be achieved by competitive blocking, i.e. preincubation of antisera raised against wMT-2 with wMT-1 antigen and vice versa. A complete loss of MT staining was observed postblocking with pretreated wMT-1 antiserum. In contrast no visual loss in intensity was detected after pretreatment of wMT-2 antiserum (Fig. 4B). This indicates isoform-specific wMT-2 accumulation in the chloragogenous tissue. As to be expected, no immunostaining was detected with either antiserum preincubation with both antigens (Fig. 4C), and similarly, no MT-positive staining was observed in control earthworms native to clean soil (Fig. 4D). Immunohistochemistry performed on posterior transverse sections of the earthworm revealed the precise cellular localization of the cadmium-responsive MT. Staining was observed in the apical cytoplasm of the intestinal epithelial cells and in the chloragogenous tissue surrounding the basal layer and the typhlosole (A and B). Higher magnification of the chloragogenous tissue indicated that the MT-positive staining was most intense either within discrete vesicles in the apical cytoplasm of intact cells abutting the coelomic cavity or as apical projections released into the coelomic cavity or typhlosole (C).

Immunogold Cytochemistry and Electron Probe X-ray Microanalysis—At the EM level, chloragogenous tissue was initially smeared onto titanium grids and air-dried (18). As described previously (22), cadmium and sulfur were co-localized within...
Thus metal binding. Furthermore, earthworms, nematodes, and snails all express two distinct MT isoforms that have analogous structure-function relationships.

Both recombinant L. rubellus isoforms exhibit the ability to bind six cadmium ions, a number that is identical to C. elegans MTs (8). Both earthworm and nematode MTs have 20 cysteine residues, yielding a stoichiometry marginally less than vertebrate MTs with their 7 metal atoms bound to proteins with a comparable number of cysteine residues. Although wMT-2 and human MT share the same cadmium retention potential during proton competition, wMT-1 is more susceptible to metal loss. This is a striking observation given the significant homology and conservation of all cysteine motifs between the earthworm isoforms. One major difference prevails, namely a 2-amino acid insertion/deletion approximately in the center of the sequence. If the earthworm protein forms a two-domain structure, similar to vertebrate MTs, this region would punctuate two domains comprising of an N-terminal Cys\(^{11}\) domain and a C-terminal Cys\(^{9}\) domain. This would result in a 6- and 4-amino acid putative link in wMT-1 and wMT-2, respectively. It has previously been shown that in vertebrates, an increase of the linker region reduces the overall stability of the MT molecule (25). Therefore, the observation that wMT-1 is less stable than wMT-2 would be consistent with a two-domain hypothesis in which the linker modulates the stability of the protein. Furthermore, partial demetallation and subsequent proteolysis of vertebrate MTs can be used to generate individual domains (12). This phenomenon is reflected in the analysis of native MTs extracted from the related compost earthworm Eisenia fetida. Not only does it have a longer linker region than L. rubellus, but sophisticated proteomic analysis could only yield the N-terminal half, equivalent to Cys\(^{11}\), of a proteolytically cleaved MT (10). However, the proteins investigated in this study, both native and recombinant, reflected the intact protein as confirmed by SDS-polyacrylamide gel electrophoresis. Overall, the limited chemical analysis has highlighted that although the ratio of metal binding is identical in both isoforms, they differ significantly in their metal binding properties, thus providing the first indication of a differential structure-function relationship.

Increased evidence for isoform-specific roles was provided by the transcript analysis. The relative expression of wMT-2 compared with wMT-1 in response to cadmium over time and dose was determined. These results conclusively show wMT-2 to be the sole cadmium-responsive isoform. Exposure to 30 \(\mu\)g of cadmium/g of soil induced a significant transcriptional response after 21 days exposure; however, 300 \(\mu\)g of cadmium/g of soil elicited a 400-fold response of after 7 days, which increased to >2500-fold after 21 days. This considerable induction is comparable with the 2000-fold induction observed when total MT transcript measurements were quantified from worms resident to natural soils containing ~600 \(\mu\)g of cadmium/g of soil (2). This indicates that the observed response is nontransient and not followed by the activation of a secondary system. Thus this suggests that wMT-2 is the primary response for these organisms to cadmium exposure. Collectively these observations uphold the notion that only wMT-2 is involved in cadmium trafficking. However, it is unlikely that every mole of cadmium is bound to MT per se as the amount of cadmium body burden within the earthworm would equate to ~1% MT protein in relation to total body mass.

The precise mechanism(s) that modulate the observed positive transcriptional regulation of wMT-2 remains elusive. In higher eukaryotes the transcription factor MTF-1 has been shown to be the major metal regulator of MT expression acting through metal responsive elements located within the MT promoter region. However, we do not know at present whether a
homologous system exists in the earthworm, especially in light of the fact that the C. elegans MT promoter lacks multiple metal responsive element sequences, and a homology search of the whole genome data base indicates the absence of a phylogenetically conserved MTF-1. Therefore, further investigation is required to determine whether the earthworm shares a novel or alternative system with its invertebrate counterpart or employs the vertebrate response mechanism.

One major advantage of the earthworms is that it is possible to visualize an entire trans-section within a single light microscopic image. This section comprises the major organs involved in absorption (the intestine), primary detoxification (the chloragog, equivalent to a vertebrate liver), and excretion (the nephridia, equivalent to a vertebrate kidney). With the aid of the wMT-2-specific polyclonal antiserum, we were able to follow the involvement of MT at key steps of cadmium transit through the cross section of a higher eukaryotic organism. Initially wMT-2 is located in the cytoplasm adjacent to the apical membranes of alimentary epithelial cells; however, it appears to have little or no involvement in the subsequent trafficking of cadmium through the epithelium and across the basal layer. Clearly another metal transporter/metal pump is involved at this step. A possible candidate may be a homologue to C. elegans cdr-1, a 32-KDa integral membrane protein shown to respond specifically to cadmium in a manner parallelling the expression of MT. In earthworms, cadmium is sequestered again by wMT-2 and compartmentalized once it enters the chloragogenous tissue. This correlates with previous electron probe analysis that identified cadmium- and sulfur-rich granules within this tissue (5). By examination of adjacent cryosections using electron microscopic ultrastructural analysis, XRNA electron probe, and immunogold labeling, we have been able to conclusively prove that these cadmium- and sulfur-rich granules are indeed concentrated compartments of wMT-2 protein. The Cd–MT sequestering compartments are released via exocytosis or shedding of the apical portions of the chloragocytes. This releases these granules either into a fold within the gut epithelium, known as the typhlosole, or directly into the coelomic cavity. In the coelom it is evident that the MT-rich granules are endocytosed by coelomocytes, wandering cells akin to vertebrate macrophages involved in immunocompetence. Finally, intense staining is observed in the nephridia (data not shown), indicating that these organs play a role in cadmium excretion although it is still unclear how the metal is delivered to them.

Does the primary sequence contain any specific feature that would explain why this isoform is linked to cadmium detoxification via a compartmentalization process? Analysis of the derived amino acid sequences identified two N-glycosylation signal sequences that are present only in wMT-2 (2). These may facilitate post-translational modification, which in turn may be instrumental in targeting this specific isoform to intracellular compartments. In addition the two isoforms exhibit differential cluster stability. This suggests that the more labile cluster of wMT-1 is better adapted to be involved as a carrier and donor of essential metals such as zinc and copper, whereas wMT-2 has evolved to scavenge and immobilize nonessential metals like cadmium. Previous work (22) has suggested that the compartment involved as the end point of accumulation may be lysosomes. Given its ability to retain its metal load at higher proton concentrations, wMT-2 is a well adapted candidate to sequester metals within acidified compartments.

In conclusion wMT-2 is the sole cadmium-responsive MT isoform in earthworms both at the transcriptional and translational level. This protein is targeted in intracellular compartments, which are involved in a high capacity detoxification pathway. Sequence and chemical analyses have provided the molecular basis underlying the specific involvement of wMT-2 in cadmium sequestration. By exploiting molecular tools, we have been able to track the involvement of MT in the complete cycle of cadmium trafficking within a higher eukaryote, which is central to soil quality and fertility.

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