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Physiological relevance and contribution to metal balance of specific and non-specific Metallothionein isoforms in the garden snail, *Cantareus aspersus*

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Abstract Variable environmental availability of metal ions represents a constant challenge for most organisms, so that during evolution, they have optimised physiological and molecular mechanisms to cope with this particular requirement. Metallothioneins (MTs) are proteins that play a major role in metal homeostasis and as a reservoir. The MT gene/protein systems of terrestrial helicid snails are an invaluable model for the study of metal-binding features and MT isoform-specific functionality of these proteins. In the present study, we characterised three paralogous MT isogenes and their expressed products in the escargot (*Cantareus aspersus*). The metal-dependent transcriptional activation of the three isogenes was assessed using quantitative Real Time PCR. The metal-binding capacities of the three isoforms were studied by characterising the purified native complexes. All the data were analysed in relation to the trace element status of the animals after metal feeding. Two of the three *C. aspersus* MT (CaMT) isoforms appeared to be metal-specific, (CaCdMT and CaCuMT, for cadmium and copper respectively). A third isoform (CaCd/CuMT) was non-specific, since it was natively recovered as a mixed Cd/Cu complex. A specific role in Cd detoxification for CaCdMT was revealed, with a 80–90% contribution to the Cd balance in snails exposed to this metal. Conclusive data were also obtained for the CaCuMT isoform, which is involved in Cu homeostasis, sharing about 30–50% of the Cu balance of *C. aspersus*. No apparent metal-related physiological function was found for the third isoform (CaCd/CuMT), so its contribution to the metal balance of the escargot may be, if at all, of only marginal significance, but may enclose a major interest in evolutionary studies.

Keywords Cadmium · Copper · Zinc · Metallothionein · Mollusca · Pulmonata

Abbreviations

AAS	Atomic absorption spectrophotometry
CaMT	*C. aspersus* MT (global denomination)
CaCdMT	Cd-specific *C. aspersus* MT isoform
CaCuMT	Cu-specific *C. aspersus* MT isoform

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CaCd/CuMT	Mixed metal *C. aspersus* MT isoform
ESI-MS	Electrospray ionization mass spectrometry
ESI-TOF MS	Time-of-flight electrospray ionization mass spectrometry

Introduction

Fluctuating environmental availability of metallic trace elements represents a constant challenge for most organisms, which have to activate regulatory mechanisms to adjust and keep their internal trace element status within a physiologically tolerable range. This holds even more so because certain metal ions exert toxic effects at even very low concentrations, whereas other metals that are essential constituents of biomolecules cause adverse effects at high concentrations. Proteins playing an accommodating role in respect to these contrasting impacts are the so-called Metallothioneins (MTs). They belong to a ubiquitous, heterogeneous family of proteins with the ability to bind closed-shell metal ions via the sulphur atoms of their cysteine residues (Kägi and Kojima 1987; Sigel et al. 2009; Blindauer and Leszczyszyn 2010). In no animal group other than pulmonate molluscs, evolutionary differentiation brought about MT isoforms that possess such a strictly exclusive, sequence-based metal specificity (Palacios et al. 2011) being involved in contrasting metal-specific tasks such as cadmium detoxification, on the one hand, and homeostatic copper regulation, on the other (Dallinger et al. 1997). Hence, a number of pulmonate species has so far been shown to respond to environmental Cd exposure by synthesising an MT isoform loaded with high amounts of Cd (Dallinger et al. 1989). Its amino acid sequence has been elucidated for several species, including the Roman snail, *Helix pomatia* (Dallinger et al. 1993), the copse snail, *Arianta arbustorum* (Berger et al. 1995), and the escargot, *Cantareus aspersus* (Hispard et al. 2008). In accordance with its tissue-specific synthesis in digestive and excretory organs (Chabicovsky et al. 2003) and because of its protective effect against Cd, its function has been assigned to the detoxification of this metal ion (Manzl et al. 2004), and it was thus named CdMT. In contrast, a second MT type of isoforms, almost exclusively associated with Cu, have been characterised and named CuMTs (Dallinger et al. 1997). These are synthesised exclusively in the so-called rhogocytes, a specific molluscan cell type in which haemocyanin, the Cu-bearing respiratory pigment of snails, is synthesised (Chabicovsky et al. 2003). Consequently, a predominantly homeostatic function in connection with the supply of Cu for haemocyanin synthesis has been suggested (Dallinger et al. 2005). Recently, a third isoform was identified, first in *C. aspersus* (Hispard et al. 2008; Schuler et al. 2008) and then in other snail species (Palacios et al. 2011). This isoform exhibits no definite metal-specificity, since it was isolated from the midgut gland of Cd-exposed snails as a complex including both Cd and Cu, and was therefore named Cd/CuMT. Interestingly, the three MT protein sequences differ only in some of their non-Cys residues, signifying that these amino acids must be crucial in determining metal specificity of each isoform (Schuler et al. 2008; Palacios et al. 2011).

In the present work, we examine the role of the three MT isoforms in the accumulation and partitioning of metal ions (Cd^{2+}, Zn^{2+}, and Cu^{+}) in pulmonate tissues, using the edible snail, *C. aspersus* (the so-called escargot), as a study object. *C. aspersus* is one of the most common terrestrial pulmonate species, with an original distribution in Southern and Western Europe (Kerney and Cameron 1979) but nowadays widespread in many parts of the world owing to anthropochorous spreading (Guiller and Madec 2010). The significance of *C. aspersus* lies in its ecological impact for nutrient fluxes in the soil (Dallinger et al. 2001). Moreover, this species has been known as a pest organism in horticulture (Godan 1979) while at the same time being appreciated as the "edible snail" in gastronomy (Chevallier 1983). Therefore, a global acknowledgement of the biochemistry and physiology of metal uptake and regulation in this species is of general interest. This goal has been tackled here using different experimental approaches. Long-term metal exposure experiments yielded information about metal accumulation in the snail midgut gland and differential patterns of transcriptional induction for the three isogenes, depending on the metal surplus (Cd and Cu) applied. The three isoforms were purified and their metal load analysed quantitatively. In addition, their metal-binding abilities were assessed by mass spectrometric

analysis of the respective metal complexes obtained by recombinant synthesis in metal-supplemented *E. coli* cultures. Our data reveal that each of the three MT isoforms contributes specifically and differentially to the metal status in snail tissues, confirming the adaptational significance of the snail MT system for coping with different trace element availabilities under fluctuating (rapidly changing) environmental conditions.

Materials and methods

Animals and rearing conditions for control and metal exposure studies

Cantareus aspersus snails were from laboratory strains of the Department of Chrono-Environment of the University of Franche-Comté (Besançon, France). About 120 individuals were kept in plastic boxes on garden soil complemented with lime powder ($CaCO_3$) at 18°C and with a photoperiod of 12:12 h. Snails were fed every third day with commercially available lettuce (*Lactuca sativa*). For metal enrichment, lettuce leaves were soaked for 1 h in Titrisol standard dilutions of $CdCl_2$ or $CuCl_2$ (Merck, Darmstadt, Germany) made up to concentrations of 1 mg Cd^{2+} l^{-1} and 3 mg Cu^{2+} l^{-1}, respectively. Resulting concentrations of Cd and Cu in metal-enriched and untreated lettuce are summarised in Table 1. Another 35 individuals were fed on lettuce supplemented with Cd^{2+} or Cu^{2+}. Additionally, 40 animals fed on untreated lettuce were used as controls. On day 0, five individuals of the control group, and on days 1, 2, 3, 5, 8, 14, and 29, five snails of each group (control, Cd^{2+} and Cu^{2+}-exposed) were sampled for RNA isolation and tissue metal analyses. Finally, for MT protein purification, two groups of 30 animals each were kept in large plastic boxes and fed for 2 weeks either with uncontaminated (control diet) or Cd-enriched lettuce leaves prepared as described above. At the end of the feeding period, all snails were sacrificed by decapitation and processed for protein purification and characterisation.

RNA isolation and reverse transcription

After dissection, ~10 mg (fresh weight) of midgut gland tissue from each individual were removed for RNA isolation, whilst the remaining midgut gland and foot tissue were used to determine Cd and Cu concentrations as described below. Individual midgut gland aliquots were homogenised (Ultra Turrax T25, IKA, Staufen, Germany) in TRIzol® reagent and RNA was subsequently isolated according to a standard protocol (Sigma, Taufkirchen, Germany). The RiboGreen® RNA Quantitation Kit from Molecular Probes (Invitrogen, Karlsruhe, Germany) was used for quantification after DNase I (Fermentas, St. Leon-Rot, Germany) digestion. 450 ng of RNA per individual were subjected to cDNA synthesis (RevertAid^{TM} H Minus M-MuLV Reverse Transcriptase, Fermentas) for subsequent isoform-specific PCR and quantitative Real Time PCR.

PCR amplification, cloning and sequencing of the cDNA of the MT isogenes

For amplification of *Cd*- and *CuMT* cDNAs, the following specific primers, designed after the *H. pomatia* orthologous sequences (Dallinger et al. 2004), were used:

CdMT-S: 5′-CTC CAT GGC AAC CAT GAG CGG AAA-3′
CdMT-AS: 5′-GCG TCG ACT TGT CCT GCG GTT ACT-3′

Table 1 Cd and Cu concentrations (means ± standard deviations; $n = 5$) in control, Cd, and Cu-enriched lettuce, measured by flame atomic absorption spectrophotometry and referred to dry weight

Metal treatment of feed	Metal concentration (μg/g)	
	Cd (μmol/g)	Cu (μmol/g)
Control lettuce	0.135 ± 0.169 (0.0012 ± 0.0015)	9.214 ± 1.970 (0.145 ± 0.031)
Cd-enriched	50.135 ± 12.253 (0.446 ± 0.109)	nd
Cu-enriched	nd	223.237 ± 46.516 (3.513 ± 0.732)

nd Not determined

CuMT-S: 5′-GTG ACC GAT GCA GTT CTT GCC ATT-3′
CuMT-AS: 5′-GCG TCG ACT TGT CGT TTA TTT GCA G-3′

PCR conditions were as follows: first denaturation at 94°C for 2 min, 39 cycles at 94°C for 20 s, 55°C for 10 s, 65°C for 40 s, and a final extension at 65°C for 10 min. Amplification of the *CdMT* cDNA from *C. aspersus* generated two PCR products of the same length, which, after cloning and sequencing, were distinguished as *CdMT* cDNA and *Cd/CuMT* cDNA. The SMART™ RACE cDNA Amplification Kit (Clontech Laboratories) was used for completion of both cDNA sequences. PCR products were cloned into pCR®4 vector (TOPO TA Cloning® Kit for Sequencing from Invitrogen) and sequenced using an AB 3130 genetic analyser (BigDye Terminator v3.1 Sequencing Kit, AB). Primer sequences for RACE PCR were as follows:

CdMT3-R-S: 5′-CAG GAG CGA GCC TTG CCA GTG TGG GAG-3′
CdMT5-R-AS: 5′-GCA AGT CTT GCA GGC GGC ACA TGT-3′
CuMT-3-R-S: 5′-TGT GAC CGA TGC AGT TCT TGC CAT TGT TCC-3′
CuMT-5-R-AS: 5′-ACT GCC ACA TTT GCA TGA TCC ACT TCC GGT-3′
CuMT-3-R (NGSP) S: 5′-TGA CGA CTG CAA GTG TGG TAG CCA ATG-3′
Cd/CuMT-3-R-S: 5′-CTA CTC CTG CCA ATG CAA CAA TGA CAC C-3′
Cd/CuMT 5-R AS: 5′-CCA GTG CGG CTA TGG GAG AGA GTG GTG A-3′

Touchdown PCR was performed using Advantage 2 polymerase (Clontech) with cycling parameters as follows: 5 cycles at 94°C for 30 s and 72°C for 3 min; 5 cycles at 94°C for 30 s, 70°C for 30 s, 72°C for 3 min; 30 cycles at 94°C for 30 s, 68°C for 30 s, 72°C for 3 min. Nested PCR was applied to amplify the *CuMT* cDNA using the following conditions: 39 cycles at 94°C for 30 s, 68°C for 30 s, 72°C for 3 min and a final extension at 72°C for 10 min. NGSP (see above) stands for nested gene specific primer.

Quantitative Real Time PCR

Quantitative Real Time PCR for the *Cd*-, *Cu*-, and *Cd/CuMT* cDNAs was performed on a 7500 Real Time PCR analyser (Applied Biosystems, Foster City, CA, USA) using Power SYBR Green. Amplicon plasmids were used to generate calibration curves for copy number analysis of Δct values for each isogene. Primers were designed with Primer Express 3.0 software (Applied Biosystems). Dissociation curves were used to elucidate the optimal primer concentrations. 2 µl cDNA were applied for Real Time detection PCR in a 20 µl approach (1× Power SYBR Green PCR Mastermix, 1× U-BSA, sense-, antisense primer). All transcripts had specific amplicon lengths (*CdMT*: 56 bp; CuMT: 74 bp; *Cd/CuMT*: 59 bp) and were amplified using the following concentrations and primers:

Cd-MTCa sense, 300 nM: 5'-GCC GCC TGT AAG ACT TGC A-3';
Cd-MTCa antisense, 900 nM: 5'-CAC GCC TTG CCA CAC TTG-3'.
Cu-MTCa sense, 900 nM: 5'-AAC AGC AAC CCT TGC AAC TGT-3'
Cu-MTCa antisense, 900 nM: 5'-CGA GCA CTG CAT TGA TCA CAA-3'
CdCu-MT sense, 900 nM: 5'-TGT GGA GCC GGC TGT TCT-3'
CdCu-MT antisense, 300 nM: 5'-CAG GTG TCA TTG TTG CAT TGG-3'

Metal analysis

Cd and Cu concentrations in midgut gland tissues (samples taken on days 0, 3, 5, 8, 14, and 29) of each individual as well as in lettuce leaf aliquots were determined by flame atomic absorption spectrophotometry (AAS). After sample drying at 60°C and weight determination, digestion was achieved in 12 ml screw-capped polyethylene tubes (Greiner, Austria) with a mixture (1:1) of nitric acid (suprapure, Merck, Darmstadt, Germany) and deionised water in a heated aluminium digestion oven at 70°C until a clear solution was obtained. The samples were diluted to 11.5 ml with deionised water. Cd and Cu concentrations were measured in the flame of an atomic absorption instrument (model 2380, Perkin Elmer, Boston, MA, USA).

Purification of native MT isoforms by standard chromatography and HPLC

After the 14-day exposure treatment (see above), midgut glands of control and Cd-exposed snails were

pooled from five animals each and processed immediately for chromatography or otherwise stored at −80°C until further use. Pooled midgut gland samples (~3.0–3.5 g fresh weight) were homogenised in a threefold volume (v/w) of 25 mM Tris HCl buffer (pH 7.5) to which 100 mM NaCl, 5 mM 2-mercaptoethanol (Merck), and 0.1 mM phenylmethylsulfonyl fluoride (Merck) had been added. Homogenates were centrifuged for 1 h at 27,000×*g* in a high speed centrifuge (model RC 5C, Sorvall Instruments, Golden Valley, MN, USA). Resulting supernatants were purified in a step by step procedure by DEAE (diethylaminoethyl) cellulose extraction, gel permeation chromatography, ultrafiltration and reversed phase high performance liquid chromatography (RP-HPLC) (Berger et al. 1997). For gel permeation chromatography, samples were split into 6-ml aliquots and applied successively to a column (15 × 300 mm) packed with Sephacryl S-100 (GE Healthcare Europe GmbH, Munich, Germany), calibrated with a mixture of Blue Dextran (2000 kDa), chicken egg albumin (45 kDa), myoglobin (18.5 kDa), and vitamin B_{12} (1.35 kDa). After chromatography, metal concentrations (Cd, Cu and Zn) were measured in fractions by AAS and the presumed MT-containing fractions were pooled and concentrated by ultrafiltration (Amicon YM1, Beverly, MA, USA; 1 kDa cut-off). Subsequently, samples were further fractionated on an HPLC system (model 501, Waters, Milford, LA, USA) equipped with a multi-wavelength detector (model 490E; Waters), using a μBondapack C_{18} column (Waters). Elution was performed in a Tris HCl/acetonitrile gradient over 35 min (Hispard et al. 2008). Fractions were diluted with deionised water and analysed for metal concentrations (Cd, Zn, Cu) as described above.

Recombinant synthesis and purification of the metal-MT complexes

The cDNAs encoding each of the three *C. aspersus* isoforms, isolated as described in the previous section, were subcloned in the pGEX expression vector for recombinant synthesis of the corresponding proteins in *E. coli* BL21 cells, essentially as described for the *H. pomatia* MT isoforms (Palacios et al. 2011). Recombinant synthesis of CdMT, CuMT and Cd/CuMT was performed in metal supplemented LB medium (300 μM $CdCl_2$ or 500 μM $CuSO_4$), which allows the recovery of in vivo folded metal-MT complexes. Full analysis of all recombinant metal complexes of *C. aspersus* MT isoforms will be provided in a forthcoming publication.

Mass spectrometry analysis of recombinant metal-CaMT complexes

Molecular mass determination was performed by electrospray ionisation mass spectrometry equipped with a time-of-flight analyser (ESI-TOF MS) using a Micro Tof-Q Instrument, Bruker Daltonics Gmbh (Bremen, Germany) calibrated with NaI (200 ppm NaI in a 1:1 H_2O: isopropanol mixture), interfaced with a Series 1100 HPLC pump (Agilent Technologies) equipped with an autosampler, both controlled by the Compass Software. The experimental conditions for analysing proteins with Cd were: 20 μl of the sample were injected through a long PEEK tube (1.5 m × 0.18 mm i.d.) at 40 μl/min under the following conditions: capillary-counter-electrode voltage, 5.0 kV; desolvation temperature, 90–110°C; dry gas, 6 l/min. Spectra were collected throughout an m/z range from 800 to 2000. The proteins containing copper were analysed by injecting 20 μl of the 30 μl/min sample; capillary-counter-electrode voltage, 4.0 kV; desolvation temperature, 80°C; m/z range from 800 to 2000. The liquid carrier was a 90:10 mixture of 15 mM ammonium acetate and acetonitrile, pH 7.0. All samples were injected at least in duplicate to ensure reproducibility. In all cases, molecular masses were calculated according to the method reported by Fabris et al. (1996).

Statistics

Statistical analyses were performed using the software packages Statistica (version 8; StatSoft Inc., Tulsa, OK, USA) and Sigma Plot (version 11; Systat Software Inc., Chicago, IL, USA). Real Time PCR plots were tested by analysis of variance (ANOVA). In addition, differences between single values of different treatments (Cd or Cu-exposed versus controls) were analysed by means of the Mann–Whitney rank sum test. A *t*-test was applied for statistical comparison of Cd and Cu concentrations in midgut gland during metal exposure. In all cases, statistical significance was defined at $P \leq 0.05$.

Results and discussion

The three MT isoforms encoded in the *C. aspersus* genome

Figure 1 shows the amino acid sequences of the three MT isoforms from *C. aspersus*, as deduced from their respective cDNA sequences (GenBank accession numbers: CaCdMT, ABL73910; CaCuMT, ABM55268; CaCd/CuMT, ABM92276; Palacios et al. 2011). Upon alignment, it became evident that the three proteins share a high degree of similarity, particularly for the highly conserved cysteines and some amino acid positions flanking them. All isoform sequences largely match those of the orthologous MT isoforms in the Roman snail (*Helix pomatia*) (Fig. 1a), previously isolated and characterised from the midgut gland of *H. pomatia* (Dallinger et al. 1993, 1997; Berger et al. 1997). The third MT isoform (CaCd/CuMT), first identified in *C. aspersus* among all snail species (Schuler et al. 2008; Hispard et al. 2008), exhibits a sequence with intermediate peculiarities between those of CaCuMT and CaCdMT (Fig. 1a) and is largely similar to the corresponding isoform in *H. pomatia* which occurs in two allelic variants (Fig. 1b). It is worth remembering, however, that the designation as CaCd/CuMT has been ascribed to this isoform not from sequence features, but owing to the fact that it is natively recovered as a protein complex including Cd^{2+} and Cu^{+} ions simultaneously (Hispard et al. 2008). The occurrence of three MT isoforms with differential metal specificities and functions seems to be an evolutionary hallmark of this mollusc taxon (Palacios et al. 2011). Although it is obvious that all three MT isoforms must interact to maintain a physiological metal balance in their host, their particular contribution to the metal status of snails has so far not been elucidated.

Metal accumulation in *C. aspersus* snails

In the present study, garden snails were exposed to elevated Cd or Cu levels in the food over a long-term period of 29 days. During this time, metal accumulation and transcriptional activation of the three MT isoforms were assessed in the snail midgut gland,

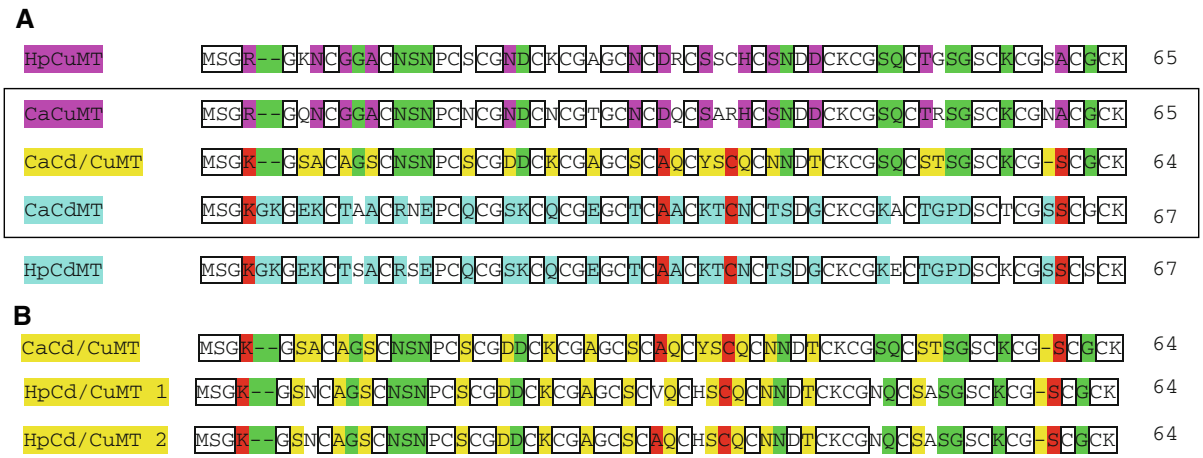

Fig. 1 **a** Amino acid sequence of three MT isoforms (in framed box) of *Cantareus aspersus*, featuring one peptide (CaCuMT) largely matching the Cu-specific isoform of *Helix pomatia* (HpCuMT) (above framed box), beside a second isoform (CaCdMT) with a high degree of similarity with the Cd-specific peptide from *Helix pomatia* (HpCdMT) (below framed box). In the middle of CaCuMT and CaCdMT (in framed box) a third isoform is shown (CaCd/CuMT) with an amino acid sequence sharing peculiarities with CaCuMT and CaCdMT. **b** Comparison of the CaCd/CuMT sequence or *C. aspersus* with two HpCd/CuMT sequences (allelic variants 1 and 2) from *H. pomatia*. Amino acids positions shared by all isoforms are framed. Identical amino acid positions shared only by part of the isoforms displayed are *colour*-underlayed as follows: *blue*, identical between CdMT isoforms (CaCdMT and HpCdMT); *pink*, identical between CuMT isoforms (CaCuMT and HpCuMT); *red*, identical between CdMT isoforms and CaCd/CuMT; *green*, identical between CuMT isoforms and CaCd/CuMT; *yellow*, only in CaCd/CuMT. The GenBank accession numbers of these sequences are EF178297.2 (CaCuMT), EF152281.1 (CaCdMT), and EF206312.1 (CaCd/CuMT). The GenBank accession numbers of the *Helix pomatia* MTs are: AF399741.1 (HpCuMT) AF399740.1 (HpCdMT), as well as ACY71053.1 (HpCd/CuMT allelic variant 1) and ACY71054.1 (HpCd/CuMT allelic variant 2)

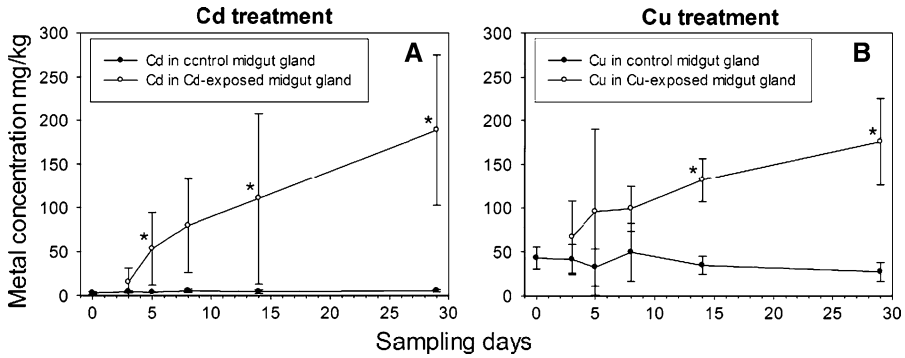

Fig. 2 Time course plot of (**a**) Cd concentration and (**b**) Cu concentration (mg/kg, dry weight) in midgut gland of control and Cd-exposed snails over 29 days. Significant differences of single values between controls and metal-exposed snails are marked by *small asterisks*. Means and two-sided standard deviations are shown for $n = 5$. The significance level for all statistical evaluations was set at $P \leq 0.05$)

since this organ accounts for most of the short and long-term metal accumulation in pulmonate snails (Dallinger and Wieser 1984; Hispard et al. 2008).

Cd was found steadily enriched in the midgut gland of Cd-exposed animals until the end of the feeding period, reaching maximum concentrations of more than 150 µg/g Cd. At the end of the exposure period, midgut gland Cd concentrations in exposed snails exceeded control levels by a factor of about 30 (Fig. 2a). In contrast to Cd, Cu uptake in the midgut gland of *C. aspersus* started from a considerably higher concentration level under control conditions (Fig. 2b) showing accumulation rates in metal-exposed individuals that are more restrained when compared to Cd accumulation. This observation coincides well with similar findings in other pulmonate snails (Dallinger and Wieser 1984) and reflects the fact that Cu is an essential trace element which has cellular concentration levels that are, to a certain extent, subject to a regime of intracellular regulation (Dallinger et al. 2005). In fact, significantly increased Cu concentrations were reached in the midgut gland of exposed snails only towards the end of the long-term feeding period, when Cu concentrations exceeded the respective control levels by a factor of 6 (Fig. 2b).

Transcription regulation of the *C. aspersus* MT isogenes

The long-term course of mRNA transcription of the *CdMT*, *CuMT* and *Cd/CuMT* genes in *C. aspersus* varied in an isoform and metal-specific manner, as shown by quantitative Real Time PCR results (Fig. 3). Already under control conditions, the basal mRNA transcription rates differed significantly between the three MT isogenes, showing the highest levels for *CdMT*, followed by intermediate levels of *CaCuMT*, and lowest values for *CaCd/CuMT* (Fig. 3). Results for gene induction are reported and discussed separately for each MT isoform.

Transcription of the CaCdMT gene

Upon Cd exposure, a significant induction of transcription was observed for *CaCdMT* (Fig. 3a), in contrast to the null response detected for the two other isogenes (Fig. 3b, c). This strong upregulation persisted until day 29, yielding mRNA copy numbers which ranged between 200,000 and 600,000, in contrast to the control group which showed copy numbers below 100,000. Interestingly, the *CaCdMT* induction rate reached a peak on day 8 of the feeding period (Fig. 3a). This may be due to the fact that at the beginning of Cd exposure, the concentration of free Cd^{2+} ions able to interact with the gene regulatory regions is higher than at later stages, when an increasing fraction of the metal ion is already bound to the CdMT protein. Overall, the transcriptional response of the *CdMT* gene in snails appears to proceed more slowly than in mammals, where highest induction peaks are observed few hours after Cd exposure, at least when injecting Cd (Swerdel and Cousins 1982). Furthermore, *MT gene* induction following intraperitoneal administration of Cd in a

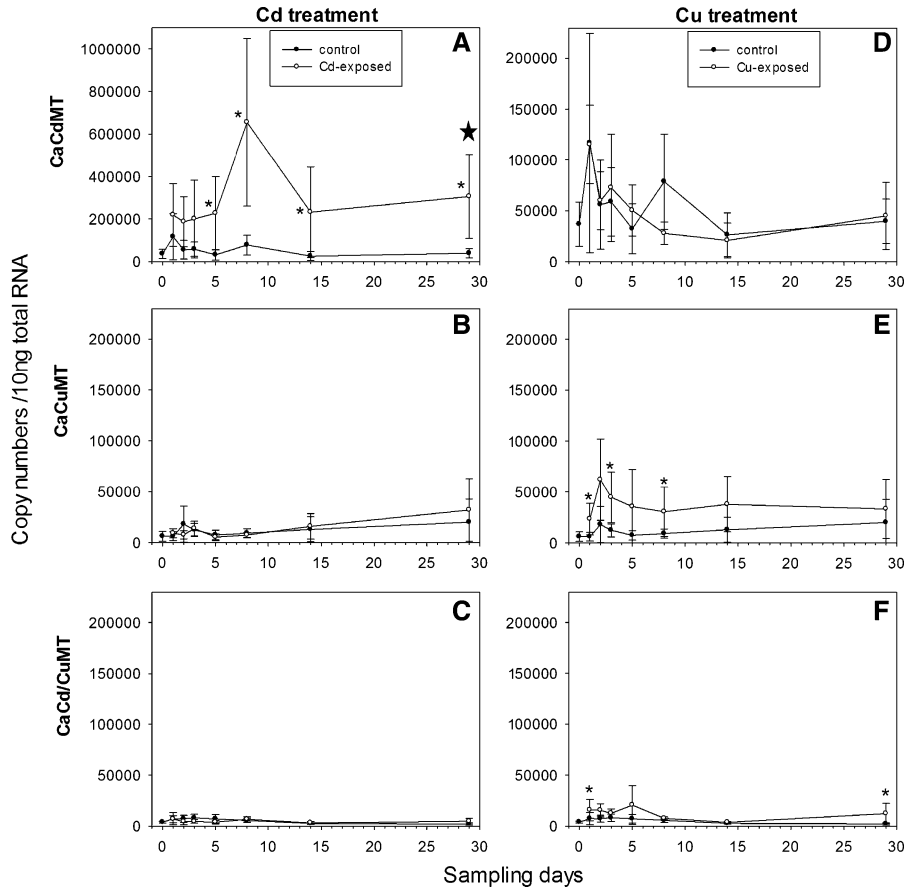

Fig. 3 Time course plots of quantitative Real Time PCR results of metal-dependent and control mRNA transcription (mRNA copy number/10 ng of total RNA) of *C. aspersus MT* isoform genes over 29 days after Cd-induction for: (**a**) *CaCdMT*, (**b**) *CaCuMT* and (**c**) *CaCd/CuMT*; and after Cu-induction for: (**d**) *CaCdMT*, (**e**) *CaCuMT*, and (**f**) *CaCd/CuMT*. Means and two-sided standard deviations are shown for $n = 3$–5 (mRNA copy numbers. Significant curves upon ANOVA are marked by a *large asterisk*. Significant differences of single values between controls and metal-exposed snails are marked by *small asterisks*. The significance level for all statistical evaluations was set at $P \leq 0.05$)

marine flatfish peaked in the liver after 4 days, and more rapidly in kidney and gills (George et al. 1996). This indicates that *MT* gene induction is tissue- and species-dependent, which may reflect differing molecular pathways and agents involved in such mechanisms (Höckner et al. 2009).

Conversely to Cd treatment, there was no induction of the *CaCdMT* gene by Cu exposure (Fig. 3d). These findings confirm that the *CaCdMT* gene of *C. aspersus* is upregulated specifically by Cd, which is consistent with the particularly important role ascribed to the CdMT protein upon Cd uptake through the feed (see below). Apart from its high response towards Cd, it cannot be excluded that *CaCdMT* may also be responsive to other, non-metallic cellular or environmental stressors, in a similar way that the orthologous gene in *H. pomatia* also exhibits a slight response towards non-metallic environmental stresses, such as desiccation (Egg et al. 2009).

Transcription of the CaCuMT gene

While there was no response of *CaCuMT* towards Cd, a slight transcriptional activation of this gene was observed after Cu intake (Fig. 3e). Although in Cu-exposed animals, the trend of transcription pattern over the course of time was not significant upon testing by ANOVA, some particular mRNA concentrations (i.e. those on days 1, 3 and 8) showed significant differences compared to the respective control values. However, in comparison with the strong responsiveness of the *CaCdMT* gene towards Cd, this occasional upregulation of the *CaCuMT* gene by Cu must be considered as very weak. Overall, this is consistent with the hypothesis that the main task of the *CaCuMT* isogene is not Cu detoxification, but rather the physiological homeostasis of this metal ion. In *H. pomatia*, the mRNA of the homologous gene is exclusively detected in rhogocytes, which are the cells where the Cu-bearing respiration pigment haemocyanin

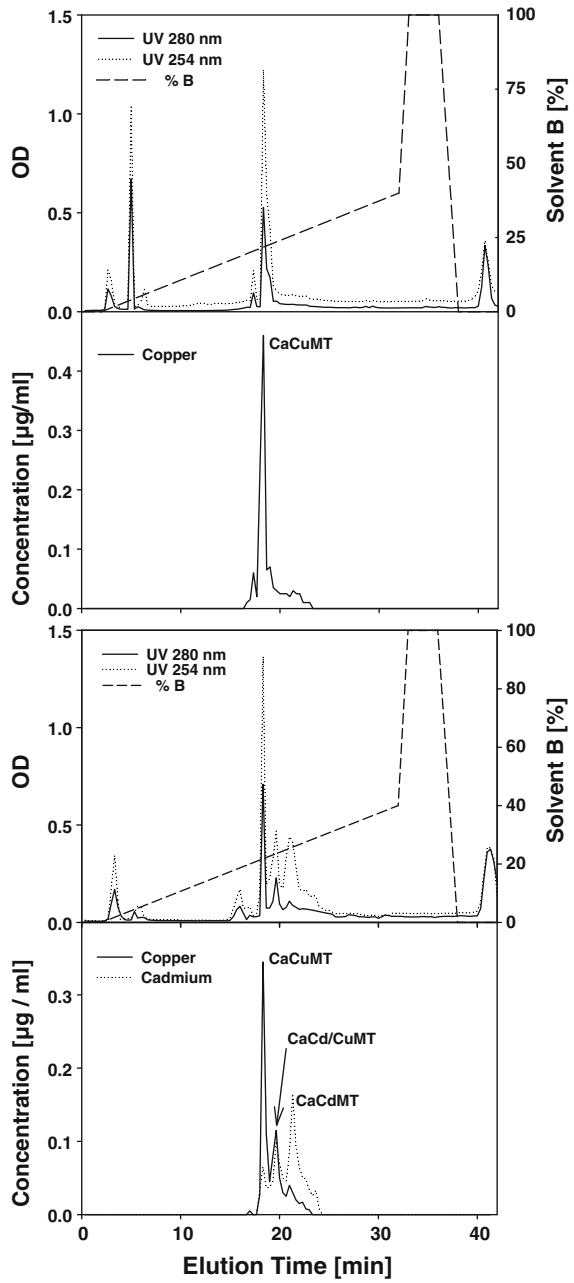

Fig. 4 Elution patterns of MT isoforms from cytosolic midgut gland homogenates of control (**a**, **b**) and Cd-exposed individuals of *C. aspersus* (**c**, **d**) upon RP-HPLC, obtained from ultrafiltrated protein extracts after initial anion exchange and gel permeation chromatography (see details in Materials and methods). **a** RP-HPLC elution profile (elution time, *x*-axis) of MT isoforms from control snail midgut glands showing the optical density (left *y*-axis) at 280 and 254 nm and the elution gradient (solvent B, right *y*-axis); (**b**) Cu concentrations in fractions of CaCuMT, measured by AAS. **c** RP-HPLC elution profile (elution time, *x*-axis) of MT isoforms from midgut glands of Cd-exposed snails showing the optical density (left *y*-axis) at 280 and 254 nm and the elution gradient (solvent B, right *y*-axis); (**d**) Cd and Cu concentrations in fractions of CaCuMT, CaCd/CuMT, and CaCdMT, measured by AAS

is synthesised. For this reason, HpCuMT has been suggested to serve in Cu donation to the nascent haemocyanin (Dallinger et al. 2005).

Transcription of the CaCd/CuMT gene

As shown by the Real Time PCR results, neither Cd nor Cu exposure induced the *CaCd/CuMT* gene (Fig. 3c, f). Noteworthy, and as for CuMT, in Cu-exposed snails a few mRNA copy number values appeared to be slightly elevated compared to the respective controls (Fig. 3f). The level of transcriptional activation of the *CaCd/CuMT* gene was the lowest observed among all three *C. aspersus MT* genes, which, together with the low rate of constitutive expression, suggests that its contribution to the overall metal balance in this species may be of only marginal significance.

Contribution of MT isoforms to metal binding in *C. aspersus*: purification of native metal-MT complexes

Midgut gland cytosol fractions of controls and Cd-exposed snails were examined for the presence of MT isoforms and their association with Cd, Cu and Zn. To this end, midgut gland homogenates were first separated into soluble and pellet fractions. The subcellular distribution analysis of Cd, Cu and Zn revealed that Cd was mainly present in the soluble cytosol (80–90%), whereas Cu and Zn were predominantly detected in pellet fractions (Cu: 60–80%; Zn: 80–85%), a distribution that is typical for the midgut gland of terrestrial pulmonate snails (Dallinger and Wieser 1984).

For MT purification, supernatants were consecutively applied to anion exchange and gel permeation chromatography, followed by ultrafiltration and RP-HPLC fractionation. As shown by the elution profiles of the final RP-HPLC step (Fig. 4), the presence of different isoforms, and the partitioning of metals among them, depended on the metal-feeding status of the animals (controls *vs.* Cd-exposed).

Control snails rendered practically one single MT peak showing a high absorption at 254 nm (Fig. 4a). Due to its exclusive Cu load and owing to its elution behaviour in comparison with the HpCuMT isoform (Berger et al. 1997; Dallinger et al. 2005), this peak was attributed to CaCuMT (Fig. 4b). The identification of this sequence as a CaCuMT isoform was corroborated by the fact that, upon recombinant synthesis in *E. coli* of the corresponding cDNA (Hispard et al. 2008), the resulting protein was a unique, homometallic Cu-loaded complex, a behaviour exhibited only by highly specific Cu-thioneins (Bofill et al. 2009). This isoform was equally detected in Cd-fed snails—without major changes in its abundance, elution time, Cu load, and Cu:Cd:Zn molar ratio (Fig. 4c, d; Table 2). The nearly exclusive Cu content of this isoform, and its independence of the state of Cd accumulation in the snail, corroborates the view that CaCuMT may be involved in the homeostatic regulation of Cu, rather than being responsible for detoxification processes (Dallinger et al. 2005; Hispard et al. 2008). This is in concordance with the observation that the transcription of *CaCuMT* in the midgut gland of *C. aspersus* does not vary as a function of supra-physiological exposure to Cd or Cu.

In addition to the CuMT isoform, the Cd-exposed snails exhibited two additional MT peaks that were not present in control animals. Both peaks showed an increased absorption at 254 nm (Fig. 4c) but differed with respect to their metal content (Fig. 4d). One of them was characterised by a clear preponderance of Cd over Cu, with a molar ratio of about 10 Cd:1 Cu and only traces of Zn (Table 2), and owing to this, it was identified as CaCdMT. The ability of this peptide to spontaneously form pure Cd complexes after recombinant expression in Cd-exposed *E. coli* cells is documented below. Interestingly, a considerable amount of *CaCdMT* mRNA had been detected in untreated snails (Fig. 3). This raises the question of why CaCdMT was not detectable in control animals at the protein level, in spite of conspicuously elevated basal mRNA concentrations. Several hypotheses could account for this apparent inconsistency, for example, that the lack of protein might be the result of inhibitory post-transcriptional regulatory mechanisms, or that, in the absence of metals, the peptide resulting from translation may be readily proteolysed in the cell, since apoMTs are known to be highly susceptible to degradation (Krezoski et al. 1988).

The second additional MT peak in the elution profile of Cd-exposed snails showed the simultaneous presence of Cd and Cu (Fig. 4d). In fact, due to this mixed metal-binding character, this isoform had been designated as CaCd/CuMT (Schuler et al. 2008; Hispard et al. 2008). The quantitative analysis of its metal content showed that Cd and Cu were present at a molar ratio of about [1 Cu:0.5 Cd], with only traces of Zn (Table 2). Its identity with the expression product of the corresponding gene (*CaCd/CuMT*) (Fig. 1) was confirmed by recombinant expression of the *CaCd/CuMT* cDNA in *E. coli*, which yields an MT peptide exhibiting non-specific metal-binding abilities, a behaviour sharply contrasting with the two metal-specific isoforms, as commented below. It is worth noting that Cd injection in marine crustaceans also yielded MT complexes including both Cd and Cu ions, as shown, for example, in the crab *C. pagurus* (Overnell and Trewhella 1979). This observation was also related to the special needs of these organisms for Cu.

Table 2 Metal content (mean ± standard deviation), expressed in μg and nmol (in brackets), in the CaCuMT, CaCdMT and CaCd/CuMT preparations obtained from control and Cd-exposed snails, as well as molar ratio (Cu:Cd:Zn) of the metal load in each case

	Metal content: μg (nmol)			Molar metal ratio
	Cu	Cd	Zn	(Cu:Cd:Zn)
Control animals				
CaCuMT	0.653 ± 0.195 (10.39)	0.000 (0)	0.012 ± 0.007 (0.18)	1:0:0.017
Cd-exposed animals				
CaCuMT	0.801 ± 0.115 (12.6)	0.087 ± 0.019 (0.77)	0.009 ± 0.001 (0.14)	1:0.06:0.011
CaCdMT	0.018 ± 0.005 (0.28)	0.316 ± 0.045 (2.81)	0.004 ± 0.002 (0.06)	1:10.03:0.18
CaCd/CuMT	0.255 ± 0.053 (4.01)	0.237 ± 0.029 (2.11)	0.005 ± 0.002 (0.08)	1:0.53:0.020

Metal contents are expressed as means and standard deviations from three repetitive elutions. Metal ratio is referred to Cu (=1)

Compared to MTs from other animal species, the *C. aspersus* native MT preparations were characterised by their remarkably poor content of Zn, even under physiological conditions (Fig. 4, Table 2) (Gehrig et al. 2000). Already in control snails, Zn was present in minor quantities in midgut gland cytosolic MT fractions and very low amounts, if any, were detectable in MT-containing fractions after gel permeation chromatography. Even less Zn, if at all, could be assessed in any of the three MT isoform peaks purified from Cd-treated snails (Table 2). Interestingly, low content or complete absence of Zn seems to be a consistent feature of pulmonate MTs and may be a consequence of the fact that in pulmonate midgut glands this metal ion is predominantly associated with granular fractions, where it may occur in a chemical form not suitable for MT binding (Dallinger et al. 1989). Therefore, the handling of essential Zn in pulmonate snails does not seem to be mediated primarily by MTs, which is concordant with the inability of Zn^{2+} to induce any of the three *C. aspersus* MT isoform genes. As shown before in this study, the snail CdMT isoform is synthesized at low basal levels in the absence of Cd^{2+}, when it may be present as Zn-loaded complexes, hardly detectable because of their low concentration. Most probably they are involved in stress-responsive functions (Egg et al. 2009) and therefore unlikely to play a major role for the Zn status in the snail organism.

Specificity of the metal-binding behaviour of the CaMT isoforms

We have recently shown that the *H. pomatia* metal-specific isoforms (HpCdMT and HpCuMT) exhibited a sequence-inherent property to form single, homometallic complexes when recombinantly synthesised in bacteria grown in media supplemented with their cognate metal and not vice versa (Palacios et al. 2011). Thus the properties of the metal-MT preparations resulting from recombinant synthesis can be considered an accurate test of MT metal-specificity. Consequently, we first characterised the products of expression of *CaCdMT* and *CaCuMT* in *E. coli* cells grown in cadmium and copper enriched media. In the former case, CaCdMT rendered a unique, homometallic cadmium complex as shown by the single ESI-MS peak of 7486.9 Da (corresponding to a Cd_6–

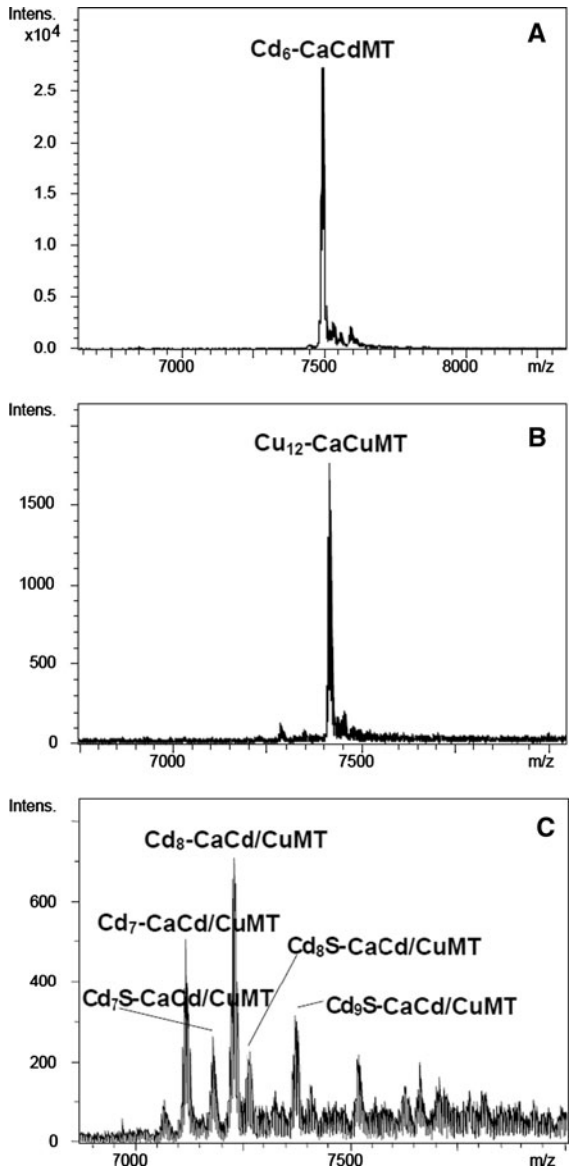

Fig. 5 Deconvoluted ESI-TOF MS spectra of different metal-MT complexes recombinantly synthesised: (**a**) CaCd-MT obtained from Cd-enriched *E. coli* cultures; (**b**) CaCuMT produced by Cu-enriched *E. coli* cultures; and (**c**) CaCd/CuMT produced by Cd-enriched *E. coli* cultures

CaCdMT complex) (Fig. 5a). In contrast, when recombinantly produced in the presence of Cu, CaCdMT is unable to form homometallic, unique complexes (manuscript in preparation). This is exactly the same result yielded by the orthologous HpCdMT, indicating that this feature of extreme Cd specificity may be shared by all pulmonate snail CdMT isoforms (Palacios et al. 2011).

On the other hand, when the *CaCuMT* gene was recombinantly expressed in *E. coli* under Cu surplus, the only recovered product was identified as a homometallic complex with a molecular mass of 7408.3 Da, corresponding to the Cu_{12}-CaCuMT species (Fig. 5b). In contrast, in this case, recombinant synthesis of this isoform with Cd or Zn produced variable mixtures of complexes with different stoichiometries, but never homometallic species (data not shown; manuscript in preparation). These results confirm the exceptional specificity of the CuMT isoform for Cu^{+}, which is also corroborated by its nearly exclusive Cu load when purified from Cd-exposed snails (Table 2). In fact, CaCuMT synthesis is not upregulated by Cu or other metals (Fig. 3b, e), as otherwise would be expected for a protein functioning as a Cu donor for haemocyanin synthesis (Dallinger et al. 2005).

The higher sequence similarity of the CaCd/CuMT isoform with CaCuMT than with CaCdMT (Fig. 1), led to the assumption that CaCd/CuMT and CaCuMT share a common ancestor in pulmonate snail origin (Palacios et al. 2011). A reflection of this close sequence resemblance is that both isoforms are natively recovered as Cu-containing complexes (homometallic or heterometallic) (Fig. 4; Table 2). But CaCd/CuMT is also associated with conspicuous amounts of Cd^{2+} at a molar ratio of [1 Cu:0.5 Cd] (Table 2), thus, in contrast to the specific CaCuMT isoform, CaCd/CuMT lacks metal specificity, either because it has not yet achieved this property or because it lost it during evolution. Indeed, the nature of the metal complexes formed by this isoform upon its recombinant synthesis under metal exposure indicates that CaCd/CuMT does not exhibit any definite metal specificity, since it gives rise to mixtures of metal:protein species with variable stoichiometries in the presence of either Cd^{2+} or Cu^{2+} (shown in Fig. 5c for the former). Furthermore, some of the Cd-species include S^{2-} ligands (Fig. 5c), which is a trait indicative of Cu-thionein character (Capdevila et al. 2005; Bofill et al. 2009; Orihuela et al. 2010). Concordantly, this isoform exhibits intermediate features regarding its induction pattern and synthesis. Hence, the *CaCd/CuMT* gene is clearly not inducible by metals, showing invariantly and extremely low mRNA levels in either control, Cu or Cd-exposed snails (Fig. 3c, f), reflecting the constitutive expression characteristics of *CuMT* genes. In contrast to this observation, a protein peak of this isoform was detectable by means of RP-HPLC in Cd-exposed snails (Fig. 4c, d), indicating that its synthesis or its formation may have been induced by the presence of Cd^{2+}. One possible explanation for these contradicting findings may be that in spite of the invariably low *CaCd/CuMT* mRNA concentration, a certain quantity of this isoform may yet be synthesised in Cd-exposed animals, so that the expressed protein can be stabilised by the presence of Cd in the corresponding complexes. The absolute amounts of this isoform in the snail may be very low. In other pulmonate species, this protein is not detectable at all after chromatographic isolation (cf. *H. pomatia*, Dallinger et al. 2005; Palacios et al. 2011). It is therefore suggested that exclusively in *C. aspersus*, CaCd/CuMT may function as a trapping molecule for excess traces of Cd and Cu during short-term events of acute exposure. Overall, its contribution to the trace element balance in the escargot may be of only marginal significance.

Conclusions

Cantareus aspersus possesses three MT isogenes: *CaCuMT*, *CaCdMT*, and *CaCd/CuMT*. As in other pulmonate snails, they have probably evolved by gene duplication from a common ancestor (Palacios et al. 2011). Only one (*CaCdMT*) of those genes is significantly upregulated by Cd, the other two genes (*CaCuMT* and *CaCd/CuMT*) are constitutively expressed and do not respond at all or only slightly to long-term Cd or Cu induction. The protein products of these genes are devoted to differential tasks, with two of them showing metal specificity for Cu (CaCuMT) or Cd (CaCdMT) and one non metal-specific isoform (CaCd/CuMT) natively binding Cd and Cu simultaneously. The CaCdMT protein binds most of the Cd absorbed by the snail tissues. Data of the present work and results from earlier studies (Hispard et al. 2008; Palacios et al. 2011) suggest that the CaCdMT isoform may account for 80–90% of the total Cd balance in snails exposed to this metal. In uncontaminated snails, this isoform is virtually absent or present at very low concentrations as a Zn complex (Egg et al. 2009). An important role for Cu balance and metabolism is attributed to the CaCuMT isoform, irrespective of whether snails are exposed to

contaminating Cu or not. This is due to the fact that the CaCuMT isoform is highly Cu-specific (see present study) binding a constant fraction of Cu absorbed by the snail tissues, mostly in rhogocytes (Dallinger et al. 2005). It is speculated that this MT-bound Cu may be available for the synthesis of the respiratory haemocyanin which represents the second significant Cu containing pool in most pulmonate snails. In addition, there are significant Cu pools present in granular cell fractions of rhogocytes, probably not in the form of a protein, and mainly in response to acute Cu exposure (Dallinger et al. 2005). Thus the share of CaCuMT in the total Cu metabolism of *C. aspersus* may be significant, although not sufficient to cover the Cu balance of the snail entirely. Its contribution to the Cu status of this species may range between 30 and 50%.

The contribution of the unspecific CaCd/CuMT isoform to the metal status of *C. aspersus* is obviously less important. This isoform may trap after Cd or Cu exposurea certain proportion of these metals. It is not present, however, under control conditions. Moreover, this isoform cannot normally be detected in other pulmonate species (Dallinger et al. 2005; Palacios et al. 2011). Because of these facts, it is suggested that its contribution to the metal status of *C. aspersus* may be low to negligible, this isoform possibly being more interesting from an evolutionary point of view. The CaCd/Cu sequence is more similar to that of CuMT isoforms than to CdMT snail isoforms. The corresponding gene is constitutively expressed, like most Cu-thionein genes, although Cd slightly enhances its transcription resulting in natively mixed Cd, Cu-complexes. Concordantly, recombinant analysis of its metal-binding behaviour reveals neither a genuine Cd- nor Cu-binding aptitude, even though some features, such as the production of sulfide-containing Cd-complexes when synthesised in cultures enriched with this metal ion, suggest a closer relationship of this isoform to Cu- than to Cd-thioneins (Bofill et al. 2009). In fact, a more detailed study (manuscript in preparation) of the structure/function relationship of this metal-binding peptide may elucidate the sequence determinants for metal specificity in MTs, i.e. which amino acids in what position favour the balance towards a Cd-thionein (divalent ion-thionein) or Cu-thionein.

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References

Berger B, Hunziker PE, Hauer CR, Birchler N, Dallinger R (1995) Mass spectrometry and amino acid sequence of two cadmium-binding metallothionein isoforms from the terrestrial gastropod *Arianta arbustorum*. Biochem J 311:951–957

Berger B, Dallinger R, Gehrig P, Hunziker PE (1997) Primary structure of a copper-binding metallothionein isoform from mantle tissue of the terrestrial gastropod *Helix pomatia* L. Biochem J 328:219–224

Blindauer CA, Leszczyszyn OI (2010) Metallothioneins: unparalleled diversity in structures and functions for metal ion homeostasis and more. Nat Prod Rep 27:720–741

Bofill R, Capdevila M, Atrian S (2009) Independent metal-binding features of recombinant metallothioneins convergently draw a step gradation between Zn- and Cu-thioneins. Metallomics 1:229–234

Capdevila M, Domenech J, Pagani A, Tio L, Villarreal L, Atrian S (2005) Zn- and Cd-metallothionein recombinant species from the most diverse phyla contain sulfide (S^{2-}) ligands. Angew Chem Int Ed Eng 44:4618–4622

Chabicovsky M, Niederstaetter H, Thaler R, Hödl E, Parson W, Rossmanith W, Dallinger R (2003) Localisation and quantification of Cd- and Cu-specific metallothionein isoform mRNA in cells and organs of the terrestrial gastropod *Helix pomatia*. Toxicol Appl Pharmacol 190:25–36

Chevallier H (1983) Recherches appliquées pour l' élevage des escargots du genre *Helix*. J Moll Stud 49:27–30

Dallinger R, Wieser W (1984) Patterns of accumulation, distribution and liberation of Zn, Cu, Cd and Pb in different organs of the land snail *Helix pomatia* L. Comp Biochem Physiol 79C:117–124

Dallinger R, Berger B, Bauer-Hilty A (1989) Purification of cadmium-binding proteins from related species of terrestrial Helicidae (Gastropoda, Mollusca): a comparative study. Mol Cell Biochem 85:135–145

Dallinger R, Berger B, Hunziker P, Birchler N, Hauer C, Kägi JHR (1993) Purification and primary structure of snail metallothionein: similarity of the N-terminal sequence with histones H4 and H2A. Eur J Biochem 216:739–746

Dallinger R, Berger B, Hunziker PE, Kägi JHR (1997) Metallothionein in snail Cd and Cu metabolism. Nature (London) 388:237–238

Dallinger R, Berger B, Triebskorn R, Köhler HR (2001) Soil biology and ecotoxicology. In: Barker G (ed) Biology of terrestrial molluscs. CAB International, Wallingford, England, pp 489–525

Dallinger R, Chabicovsky M, Berger B (2004) Isoform-specific quantification of metallothionein in the terrestrial gastropod *Helix pomatia* I. Molecular, biochemical, and methodical background. Environ Toxicol Chem 23:890–901

Dallinger R, Chabicovsky M, Hödl E, Prem C, Hunziker P, Manzl C (2005) Copper in *Helix pomatia* (Gastropoda) is regulated by one single cell type: differently responsive metal pools in rhogocytes. Am J Physiol 189:R1185–R1195

Egg M, Höckner M, Chabicovsky M, Brandstätter A, Schuler D, Dallinger R (2009) Structural and bioinformatic analysis of the Roman snail Cd-Metallothionein gene uncovers molecular adaptation towards plasticity in coping with multifarious environmental stress. Mol Ecol 18: 2426–2443

Fabris D, Zaia J, Hathout Y, Fenselau C (1996) Retention of thiol protons in two classes of protein zinc coordination centers. J Am Chem Soc 118:12242–12243

Gehrig P, You C, Dallinger R, Gruber C, Brouwer M, Kägi JHR, Hunziker PE (2000) Electrospray ionization mass spectrometry of zinc, cadmium and copper metallothioneins: evidence for metal binding cooperativity. Prot Sci 9:395–402

George SG, Todd K, Wright J (1996) Regulation of metallothionein in teleosts: induction of MTmRNA and protein by cadmium in hepatic and extrahepatic tissues of a marine flatfish, the turbot (*Scophthalmus maximus*). Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 113:109–115

Godan D (1979) Schadschnecken und ihre Bekämpfung. Ulmer, Stuttgart (Germany)

Guiller A, Madec L (2010) Historical biogeography of the land snail *Cornu aspersum*: a new scenario inferred from haplotype distribution in the Western Mediterranean basin. BMC Evol Biol 10:18

Hispard F, Schuler D, de Vaufleury A, Scheifler R, Badot PM, Dallinger R (2008) Metal distribution and metallothionein induction after cadmium exposure in the terrestrial snail *Helix aspersa* (Gastropoda, Pulmonata). Environ Toxicol Chem 27:1533–1542

Höckner M, Stefanon K, Schuler D, Fantur R, de Vaufleury A, Dallinger R (2009) Coping with cadmium exposure in various ways: the two helicid snails *Helix pomatia* and *Cantareus aspersus* share the metal transcription factor-2, but differ in promoter organization and transcription of their Cd-metallothionein genes. J Exp Zool A Ecol Genet Physiol 311:776–787

Kägi JHR, Kojima Y (1987) Chemistry and biochemistry of metallothionein. In: Kägi JHR, Kojima Y (eds) Metallothionein II. Exp Suppl 52. Birkhäuser Verlag, Basel, pp 25–61

Kerney MP, Cameron RAD (1979) A field guide of the land snails of Britain and North-west Europe. Wm. Collins & Sons, Glasgow

Krezoski SK, Villalobos J, Shaw CF, Petering DH (1988) Kinetic lability of zinc bound to metallothionein in Ehrlich cells. Biochem J 255:482–491

Manzl C, Krumschnabel G, Schwarzbaum PJ, Dallinger R (2004) Acute toxicity of cadmium and copper in hepatopancreas cells from the Roman snail (*Helix pomatia*). Comp Biochem Physiol C 138:45–52

Orihuela R, Monteiro F, Pagani A, Capdevila M, Atrian S (2010) Evidence of native metal-(S^{2-})-MT complexes confirmed by the analysis of Cup1 divalent metal ion binding properties. Chem A Eur J 16:2363–12372

Overnell J, Trewhella E (1979) Evidence for the natural occurrence of (cadmium, copper)-metallothionein in the crab *Cancer pagurus*. Comp Biochem Physiol 64C:69–76

Palacios O, Pagani A, Pérez-Rafael S, Egg M, Höckner M, Brandstätter A, Capdevila M, Atrian S, Dallinger R (2011) Shaping mechanisms of metal specificity in a family of metazoan metallothioneins: evolutionary differentiation of Mollusc MTs. BMC Biol 9:4

Schuler D, Dallinger R, Hispard F, De Vaufleury A (2008) Biochemical characterization of metallothionein isoforms in terrestrial snails: relationship between amino acid sequence and metal binding specificity. Comp Biochem Physiol 151A:S20

Sigel A, Sigel H, Sigel RKO (eds) (2009) Metal ions in life sciences; vol 5: metallothioneins and related chelators. Royal Society of Chemistry, Cambridge, UK

Swerdel MR, Cousins RJ (1982) Induction of kidney metallothionein and metallothionein messenger RNA by zinc and cadmium. J Nutr 112:801–809