Primary structure of a copper-binding metallothionein from mantle tissue of the terrestrial gastropod *Helix pomatia* L.

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A novel copper-binding metallothionein (MT) has been purified from mantle tissue of the terrestrial snail *Helix pomatia* using gel-permeation chromatography, ion-exchange chromatography and reverse-phase HPLC. Copper was removed from the thionein by addition of ammonium tetrathiomolybdate. The resulting apothionein (molecular mass 6247 Da) was S-methylated and digested with trypsin, endoproteinase Arg-C and endoproteinase Lys-C. Amino acid sequences of the resulting peptides were determined by collision-induced dissociation tandem MS. The protein is acetylated at its N-terminus, and consists of 64 amino

acids, 18 of which are cysteine residues. A comparison with the cadmium-binding MT isolated from the midgut gland of the same species shows an identical arrangement of the cysteines, but an unexpectedly high variability in the other amino acids. The two MT isoforms differ in total length and at 26 positions of their peptide chains. We suggest that the copper-binding MT isoform from the mantle of *H. pomatia* is responsible for regulatory functions in favour of copper, probably in connection with the metabolism of the copper-bearing protein, haemocyanin.

INTRODUCTION

Metallothioneins (MTs) are small cysteine-rich proteins which have a high affinity for certain heavy-metal ions, and whose synthesis is regulated, apart from intracellular metal concentrations, by a variety of hormones and xenobiotics [1,2]. Through multiple meta-thiolate co-ordination, MTs chelate metal ions such as Cd(II), Zn(II) and Cu(I). Vertebrate MTs normally bind seven atoms of Cd or Zn, and 12 atoms of Cu per protein molecule. The metals are localized in two polynuclear clusters in two distinct domains [3]. Affinity, stoichiometry and cluster specificity of the different metals bound to MT are important features characterizing the physiological performance of this protein. Its biological function is still controversial, and new data, based on mouse models either overexpressing or not expressing MT genes, support the concept of MTs as multifunctional proteins, involved in detoxification of Cd and the accumulation and distribution of Zn [4,5].

Most species express different isoforms of MT. The isoforms within a species are often similar to each other, varying at only a few positions in their amino acid chain between the conserved cysteine residues [6,7]. Differential gene regulation and distinct structural characteristics of the isoproteins are starting points for explaining the existence of multiple MT isoforms. Different functions of MT may thus result from different time patterns in MT gene expression, as well as inducer- or tissue-specific regulation of MT synthesis [8,9]. Alternatively, structurally different MT isoforms may have different functions in the same organism, tissue or cell. The best example of a structurally different MT isoform showing a specific function is MT-III, which was identified as a cellular growth inhibitory factor involved in Alzheimer's disease [10].

In spite of the supposed involvement of MT in metal metabolism, only a few examples are so far known showing how these proteins may be able to fulfil simultaneously metal-specific functions such as detoxification of toxic metal ions on the one hand and trace-element regulation on the other. One mechanism that probably enables MTs to function in such a metal-specific manner is based on the two-cluster arrangement of metals in their metal-binding domains, with a non-uniform affinity and hence different distribution of Cd(II), Zn(II) and Cu(I) between the two clusters [11]. In this case different metal-specific functions are performed by one single MT isoform. A clear metal-specific sharing of functions between different MT isoforms appears not to exist in vertebrates, but may be of importance during embryogenesis of sea urchins [12].

Here we report the primary structure of a MT isoform from the mantle tissue of the terrestrial snail *Helix pomatia* which is exclusively loaded with copper. Besides this uncommon metal-binding characteristic, this isoform also shows unexpectedly large differences when compared with a Cd-binding MT isoform, which was previously purified from the midgut gland of the same species [13]. It is suggested that the Cu-binding MT isoform from the snail's mantle is involved in the regulation of copper, which is an essential constituent of the oxygen-carrying protein, haemocyanin.

MATERIALS AND METHODS

Chemicals, reagents and enzymes

Tris-(2-carboxyethyl)phosphine hydrochloride (TCEP) was from Pierce (Rockford, IL, U.S.A.), and ammonium tetrathiomolybdate (TTM) from Aldrich (Milwaukee, WI, U.S.A.).

Abbreviations used: CID, collision-induced dissociation; DTT, dithiothreitol; ES-MS, electrospray mass spectrometry; MS/MS, tandem mass spectrometry; MT, metallothionein; TCEP, tris-(2-carboxyethyl)phosphine hydrochloride; TFA, trifluoroacetic acid; TTM, ammonium tetrathiomolybdate.

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Trypsin (EC 3.4.21.4) from bovine pancreas, endoproteinase Lys-C (EC 3.4.21.50) from *Lysobacter enzymogenes* and endoproteinase Arg-C (EC 3.4.22.8) from *Clostridium histolyticum* were all sequencing grade from Boehringer (Mannheim, Germany). All other chemicals and reagents were from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO, U.S.A.).

Animals

Roman snails (*H. pomatia* L.) were obtained from a commercial dealer and were reared at 18 °C in boxes with soil substrate. Three times a week the animals were fed with lettuce. Forty snails were killed and their mantles were dissected. Three or four organs (approx. 4 g) were pooled to one sample, and each was stored at -70 °C.

MT purification

For all chromatographic separation steps, a Waters HPLC system (model 501) equipped with a multiwavelength detector (model 490E) was used.

Each pool of mantle tissues (see above) was homogenized in 3 vol. of 25 mM Tris/HCl buffer, pH 7.5, containing 20 mM 2-mercaptoethanol and 0.1 mM PMSF and centrifuged for 20 min at 27000 g. The resulting supernatant was applied to a gel-permeation chromatography column (Sephacryl S-100; 15 mm × 30 cm) which had been calibrated by applying Blue Dextran (2000 kDa), chicken egg albumin (45 kDa), myoglobin (18.5 kDa), rabbit MT-1 (6.5 kDa) and vitamin B₁₂ (1.35 kDa). Elution was performed with a 25 mM Tris/HCl buffer, pH 7.5, containing 10 mM 2-mercaptoethanol, at a flow rate of 3 ml/min. In all sample fractions, A₂₈₀ and A₂₅₄ and concentrations of Cu, Cd and Zn were measured. MT-containing fractions were pooled, applied to an ion-exchange column (HiTrap Q, Pharmacia; column volume 1 ml), and eluted for 60 min at a flow rate of 0.8 ml/min using a gradient from 100% solvent A (10 mM Tris/HCl, pH 8), containing 1 mM dithiothreitol (DTT), to 100% solvent B (400 mM Tris/HCl, 1 mM DTT, pH 8). Cu-containing fractions were pooled and concentrated by ultrafiltration (Amicon YM1; 1 kDa cut-off) to one-fifth of the original volume. Aliquots of these concentrates (500 µl each) were fractionated using a µBondapak C₁₈ reverse-phase column (3.9 mm × 300 mm; 10 µm particle size; 12.5 nm pore size). Elution was performed over 30 min at a flow rate of 1 ml/min, using a gradient of 0–40% solvent B (25 mM Tris/HCl, 60% acetonitrile, pH 7.5) in solvent A (25 mM Tris/HCl, pH 7.5). MT-containing fractions were collected manually and stored at -70 °C.

Removal of Cu and cysteine modification

To obtain apothionein, Cu was removed from MT with TTM: 100 µl of a 2 mM TTM solution were added to 4 µl of the pooled fractions from reverse-phase HPLC, and mixed by stirring for 5 min. To bind the Cu-TTM complex, 1 µl of a suspension of DEAE-Sephacel (66%, v/v) was added to the sample, mixed thoroughly, and subsequently removed by filtration using a Duran fritted glass filter, pore size 10–16 µm (Schott). The resulting solution was immediately acidified with 1% trifluoroacetic acid (TFA) and applied to a Sephadex G-25 desalting column (15 mm × 20 cm). Elution was carried out at a flow rate of 3 ml/min with 0.1% TFA. The resulting protein fractions were dried in a vacuum concentrator (Savant instruments, SpeedVac System I). For amino acid sequencing, the apothionein was modified by S-methylation as described by Hunziker [14], using 10 mM TCEP as the reducing agent.

Endoproteinase digestion and peptide mapping

The S-methylated protein was digested with trypsin, endoproteinase Lys-C and endoproteinase Arg-C, following the instructions of the supplier. Resultant peptides were recovered by reverse-phase HPLC (Waters model 501) using a µBondapak C₁₈ reverse-phase column (3.9 mm × 300 mm; 10 µm particle size; 12.5 nm pore size). The injected volume was 500 µl. Lys-C and Arg-C peptides were eluted over 55 min at a flow rate of 0.6 ml/min using a gradient of 0–60% solvent B (solvent A containing 60% acetonitrile) in solvent A (0.1% TFA). Tryptic peptides were recovered by eluting the sample with the same solvents as described above, but using a gradient of 0–25% solvent B over 30 min, and 25–45% solvent B over 55 min.

MS analysis

The relative masses of the total S-methylated protein and proteolytic fragments were determined by electrospray MS (ES-MS) using an API III triple-quadrupole instrument (Sciex). The collected peptides were freeze-dried and re-dissolved in an appropriate volume of 0.1% (v/v) acetic acid with 50% (v/v) acetonitrile, and were injected into the ion source of the mass spectrometer at a flow rate of 5 µl/min. Amino acid sequences of the peptides were characterized by collision-induced dissociation (CID) tandem MS (MS/MS) using argon as the collision gas at a thickness of approx. 4×10^{14} molecules/cm².

Metal analyses

Metal concentrations of fractions derived from chromatographic separations were determined by atomic absorption spectrophotometry (Perkin-Elmer, model 3280) with deuterium background correction.

Alignment of protein sequences

Comparisons between amino acid sequences were carried out by the Clustal W program [15]. The Dayhoff PAM 250 matrix was used to weight differentially different pairs of aligned amino acids [16].

RESULTS AND DISCUSSION

Protein purification

A Cu-binding MT isoform was purified from supernatants of homogenized mantle-tissue of *H. pomatia* by means of subsequent chromatographic separation steps (Figures 1–3). Since Cu-containing MTs are readily oxidized it was necessary to minimize the duration of the purification procedure. In the present work the Cu-binding MT proved to be stable for up to 2 days at 4 °C in a buffer containing DTT or 2-mercaptoethanol without purging the solution with nitrogen or argon. All purification steps for each of the pooled tissue samples were therefore carried out on 2 consecutive days. After gel-filtration chromatography of mantle supernatants, two Cu-containing components were obtained (Figure 1). The elution volume of the first of these Cu-containing peaks corresponded to high-molecular-mass fractions, and is assumed to contain predominantly the Cu-binding oxygen-carrying protein, haemocyanin. The second Cu peak appeared to consist of Cu-MT, containing only traces of Cd and Zn (Figure 1). Indeed, the molar proportions of Cu/Zn/Cd in these fractions were 100:6:1. The Cu-containing MT fractions were pooled and subsequently applied to an anion-exchange column, from which the total amount of MT was eluted in a single peak (Figure 2). After concentration of these fractions by ultrafiltration, the

Figure 1 Gel-permeation chromatography (Sephacryl S-100; Pharmacia) of mantle supernatant from *H. pomatia* showing A_{280} and A_{254} (a) and metal concentrations (b)

The MT-containing fractions are marked in (a).

Figure 2 Ion-exchange chromatography (HiTrap Q; Pharmacia) of the mantle MT isoform, derived from pooled fractions of gel-permeation chromatography (see Figure 1), showing A_{280} (—) in a gradient of solvent B (----) (a) and Cu concentration (b)

resulting MT was further purified by reverse-phase HPLC, yielding one distinct Cu-binding component, which was used for further sequence analysis (Figure 3). As shown by ES-MS analysis

Figure 3 Reverse-phase HPLC (μ Bondapak C₁₈; Waters) of the mantle MT isoform, derived from pooled and concentrated fractions after ion-exchange chromatography (see Figure 2), showing A_{280} (—) in a gradient of solvent B (----) (a) and Cu concentration (b)

(see Table 2), the resulting peptide was the only molecular ion contained in the respective HPLC fractions.

The nearly exclusive presence of Cu in the isolated MT was surprising, since MTs that bind Cu almost exclusively have so far rarely been reported. In fact, in most adult animals MT is usually occupied by Zn and Cd. In vertebrates, elevated levels of Cu-MT occur only during early development, after exposure to Cu or as a result of diseases of Cu metabolism such as Wilson's disease [17–19]. A Cu-MT was also observed during the intermolt stage in marine crustaceans, being probably involved in reconstitution processes of the Cu-containing protein, haemocyanin [20,21]. In the yeast *Saccharomyces cerevisiae* two distinct Cu-MT isoforms were identified, playing different roles in Cu detoxification and Cu metabolism [22]. Possible functions of Cu-MTs in the intracellular metabolism have recently been reviewed by Brouwer [23].

Copper removal

To remove Cu from the purified MT the molybdenum-containing reagent TTM was used. This chemical was also applied in an earlier study to remove Cu selectively from MT in a saturation procedure for MT quantification [24]. However, excessive addition of TTM results in the formation of an insoluble polymer of the Cu-TTM complex [19,25]. To avoid polymerization the soluble complex was removed by addition of DEAE-Sephacel and subsequent filtration. To avoid oxidation of the resulting apothionein, the remaining solution was immediately acidified by addition of TFA.

Mass determination and amino acid sequencing

After Cu removal and S-methylation, an aliquot of the intact protein was subjected to mass determination by ES-MS. The

Table 1 Sequences and relative masses of proteolytic peptides of the Cu-binding MT isoform from mantle of *H. pomatia* after digestion with trypsin (T1–T6), endoproteinase Lys-C (L1–L5) and endoproteinase Arg-C (A1–A3)

All peptide sequences were derived by CID-MS/MS. The peptide T2 was also sequenced by Edman degradation. Relative masses were reported as theoretically expected (Expected) from sequence determination or from comparison with sequences of respective peptides (T1–T6, L1–L5) of the already known Cd-binding MT isoform from midgut gland of the same species [13], and as determined (Found) by ES-MS in the proteolytic peptides of the present study. To improve comparability the expected relative masses of the peptides were calculated by including the mass of *S*-methylcysteine. n.d., not determined; Ac-, N-terminal acetylation.

Peptide	Sequence	Relative masses	
		Expected	Found
T1	Ac-SGR	360.2	360.2
T2	NCGGACNSNPCSGNDCK	1815.7	1815.7
T3	CGAGCNCDR	939.3	939.4
T4	CSSCHCSNDDCK	1356.5	1356.4
T5	CGSQCTGSGSCK	1158.5	1158.7
T6	CGSACGCK	769.3	769.8
L1	Ac-SGRGK	545.3	545.5
L2	NCGGACNSNPCSGNDCK	1815.7	1817.0
L3	n.d.	2279.6	2279.3
L4	n.d.	1158.5	1158.7
L5	n.d.	769.3	769.5
A1	n.d.	360.2	360.2
A2	n.d.	2924.3	2924.1
A3	n.d.	3250.8	3250.5

Figure 4 Separation of tryptic peptides of the *S*-methylated MT by reverse-phase HPLC, showing A_{220} (—) in a gradient of solvent B (----). The tryptic peptides (T1–T6) are numbered according to their position in the whole protein (see Figure 5).

relative mass obtained (Table 2) was the only one detected in the respective protein fraction, which can therefore be regarded as being homogeneous. The remaining protein sample was digested by trypsin, Lys-C and Arg-C. The resulting peptides were fractionated by reverse-phase HPLC. As shown in Figure 4, the elution profile of tryptic peptides yielded five peaks, containing six different peptides (T1–T6). Relative masses of the peptides obtained after trypsin, Lys-C and Arg-C digestions were also determined by ES-MS (Table 1). Subsequently, CID-MS/MS spectra of trypsin and Lys-C peptides in the molecular mass range 360–1817 Da were acquired. These CID spectra yielded

Figure 5 Overlaps of proteolytic peptides (horizontal lines) of the MT isoform from mantle of *H. pomatia*, recovered after digestion with endoproteinase Lys-C (L1–L5), Arg-C (A1–A3) and trypsin (T1–T6)

The acetylation of the N-terminal serine is indicated by *. Sequenced peptides are shown as solid lines, and peptides for which just relative masses were determined are marked as dashed lines. All peptide sequences were derived by CID-MS/MS. The peptide T2 was also sequenced by Edman degradation.

Table 2 Relative mass of the intact *S*-methylated protein compared with the sum of relative masses of proteolytic peptides, that cover the total length of the expected polypeptide chain as shown in Figure 5

All masses were determined by ES-MS. The relative mass of the intact *S*-methylated protein was the only one detected in the respective sample.

Peptide	Relative mass
Total protein	6499.6
L1 + L2 + L3 + L4 + L5	6498.0*
A1 + A2 + A3	6498.8*

* The sum of relative masses was corrected by subtracting the respective mass of water for each additional peptide bond.

ion series, which allowed deduction of complete amino acid sequences of the involved peptides (Table 1). Taken together, these peptide sequences matched the molecular mass of the entire MT (Table 2). The strategy of sequence determination was based on the alignment of overlapping sequences of trypsin and Lys-C peptides, and on the expected homology with the already known MT isoforms from the midgut of *H. pomatia* [13] and *Arianta arbustorum* [26] (Figure 5). Moreover, relative masses of Arg-C and Lys-C peptides as well as of the whole protein were used to confirm the accuracy of the alignment (Table 2). The amino acid chain of the whole Cu-binding MT contains neither leucine nor isoleucine. Distinction between the isobaric amino acids glutamine and lysine was mainly based on the specificity of trypsin and Lys-C. Confirmatory evidence for the proposed sequence was obtained by Edman sequencing of peptide T2 and by amino acid analyses (results not shown). The N-terminus of the protein was found to be blocked for Edman sequencing. Analysis of the corresponding N-terminal tryptic and Lys-C peptides (T1 and L1) by MS and amino acid analyses showed that the N-terminal amino acid residue is an acetylated serine. Such modification of the N-terminal serine was also found in MT isoforms isolated from midgut gland of the same species and the related species *A. arbustorum* [13,26].

Sequence comparisons

The Cu-MT isoform from the snail's mantle consists of 64 amino acids, 18 of which are cysteine residues (Figure 5). Its primary structure identifies this MT isoform as a member of class I MTs [27]. The cysteine residues of the protein are arranged in seven Cys-X-Cys motifs (in which X denotes any amino acid except cysteine). A comparison with the Cd-binding MT isoform isolated

Figure 6 Sequence comparison of MTs from different mollusc species

The sequences were aligned by means of the program Clustal W. **(a)** Intra- and inter-specific comparison of snail MT sequences: the amino acid sequence of the midgut gland MT isoform from *H. pomatia* [13] (*H.p.* Cd-MT) is compared with those of the mantle MT isoform from the same species (this study) (*H.p.* Cu-MT) and that of MTb from the midgut gland of *A. arbustorum* [26] (*A.a.* Cd-MT). Gaps are indicated by dots and identical amino acids are highlighted by a vertical line. **(b)** Alignment of MT sequences from the mussel *Mytilus edulis* [28], the oyster *Crassostrea virginica* [29] and the Cu-binding MT isoform from *H. pomatia* (Snail). Gaps are indicated by dots and identical cysteine residues are highlighted by a vertical line.

from the midgut gland of the same species shows that in both isoforms the arrangement of the cysteine residues is conserved, but an unexpectedly high number of other amino acids is exchanged (Figure 6a). The two MT isoforms differ from each other in their total length (66 amino acids for the midgut gland MT isoform and 64 for the mantle MT isoform) and at 26 positions of their peptide chains. Thus only 58% of all amino acids, or 43% of the amino acids besides the cysteine residues, are identical. In contrast with this low intraspecific similarity between the two MT isoforms of *H. pomatia*, a sequence comparison of Cd-binding isoforms from *H. pomatia* and the related species, *A. arbustorum*, reveals an identity of 86% (including cysteine residues) in amino acid positions between isoforms of both species [13,26] (Figure 6a). When sequences of snail MTs are compared with those from mussels and oysters, a high variability in both the length and composition of amino acid chains can be observed (Figure 6b). Despite these differences the positions of cysteine residues appear to be conserved to a much higher extent than those of other amino acids. By introducing two gaps, it is possible to align 16 of the 18 cysteine residues and 6 of the seven Cys-X-Cys motifs of the respective proteins. This observation suggests a strong constraint on the distribution of cysteine in molluscan MTs similar to that found in MTs from other animal groups. It has been shown that alterations in the arrangement of the cysteine residues along the polypeptide chain can dramatically decrease the capacity of the proteins for metal chelation and cadmium detoxification [30].

A comparison of the primary structure between the Cu-binding and the Cd-binding MT isoforms from *H. pomatia* reveals, as a striking feature, the replacement of several amino acid positions with asparagine residues in the Cu-binding isoform (six residues compared with one residue in the Cd-containing isoform). Moreover, the Cu-containing isoform contains fewer lysine, glutamic acid and threonine residues than the midgut gland MT isoform. Changes in the bioactivity of MTs were observed after exchanging amino acids other than cysteine, e.g. serine and lysine [31,32]. The differences in the primary structure of the two MT isoforms from *H. pomatia* therefore also suggest distinct features of the two proteins in metal-binding and physiological functioning, but this has to be supported by additional experiments.

The histidine residue at position 39 of the mantle MT isoform is remarkable by being part of one of the Cys-X-Cys motifs. Histidine is a potential ligand for metal ions and is typically lacking in class-I MT sequences. However, this amino acid can be found in a few class-II MTs, such as, for instance, in the Cu-binding MT of the yeast *Saccharomyces cerevisiae* [33]. Moreover, Cys-His-Cys motifs have been found in MTs of the nematode *Caenorhabditis elegans* [32] and the yeast *Candida glabrata* [35], as well as in a non-MT Cd-binding protein, with a molecular mass of 25 kDa, from the oligochaete *Enchytraeus buchholzi* [36]. NMR studies on the Cu-binding MT of *S. cerevisiae* showed that the histidine by itself does not participate in metal binding and that, as known from all other MTs, the cysteine residues are exclusively involved in ligand formation with metal ions [37]. Therefore direct involvement of the histidine of the mantle MT isoform in metal-binding and cluster formation is unlikely.

Functional considerations

The fact that the mantle MT isoform of *H. pomatia* is exclusively occupied by Cu suggests the involvement of this protein in the metabolism of Cu and particularly in the synthesis of the Cu-containing protein, haemocyanin. Animals possessing haemocyanin are very suitable for studying the presumed function of MT in the metabolism of Cu [23]. Re-activation of apohaemocyanin by MT through a glutathione-mediated transfer of Cu ions has been demonstrated in the american lobster *Homarus americanus* [38]. In addition, an essential role for MT in the detoxification of Cu during ecdysis of the blue crab, *H. americanus*, was suggested. During this process haemocyanin releases large amounts of reactive Cu^{2+} ions [21]. Apart from certain arthropod species, haemocyanins are also found in molluscs such as the terrestrial gastropod *H. pomatia* [39]. In fact, the so-called pore cells in the mantle of helix snails have been reported as being sites of haemocyanin synthesis [40].

H. pomatia is known to accumulate high amounts of Cd and Cu, but these two metals follow different pathways of accumulation [41–43]: Cd is mainly stored in the midgut gland, whereas most of the Cu accumulates in the foot and mantle tissue. In the midgut gland, most of the Cd is bound to an MT isoform of known primary structure [13]. In contrast with this,

most of the cytosolic Cu in the snail's mantle is bound to high-molecular-mass ligands, probably representing haemocyanin, and to the MT isoform, the primary structure of which is presented here. It has been suggested that the distinct preferences of the two MT isoforms for Cd and Cu in midgut gland and mantle of *H. pomatia* may serve different functions in Cd and Cu metabolism of this species [44].

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