Biochem. J. (1995) 311, 951–957 (Printed in Great Britain)

Mass spectrometry and amino acid sequencing of two cadmium-binding metallothionein isoforms from the terrestrial gastropod *Arianta arbustorum*

Burkhard BERGER,* Peter E. HUNZIKER,† Charles R. HAUER,‡ Neil BIRCHLER† and Reinhard DALLINGER*§

*Institut für Zoologie und Limnologie (Abteilung Ökophysiologie) der Universität Innsbruck, Technikerstrasse 25, A-6020 Innsbruck, Austria, †Biochemisches Institut der Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland, WCLR Clinical Science, Empire State Plaza, Albany, NY 12201–0509, U.S.A.

1. Two cadmium-binding metallothionein (Mt) isoforms, called Mta and Mtb, were isolated from terrestrial snails (*Arianta arbustorum*), using various chromatographic techniques, such as gel-permeation chromatography and reversed-phase HPLC. The purified proteins were S-methylated and cleaved by means of different enzymes (trypsin, endoproteinase Glu-C, and endoproteinase Asp-N). Amino acid sequences were determined by automated Edman degradation and collision-induced dissociation (CID) tandem MS. According to their primary structures, both isoforms should be attributed to class-I Mts. 2. The two forms are structurally identical, differing only by one amino acid exchange in position 60 of the peptide chain. Both isoproteins consist of 66 amino acids, 18 of which are cysteine residues. Most of the cysteine residues are arranged in seven Cys-Xaa-Cys motifs. Mta and Mtb possess an N-terminal acetylated-serine residue and contain a short N-terminal motif which shows a high degree of similarity with the N-termini of histones H4 and H2A. 3. A comparison of Mta and Mtb with other invertebrate Mts shows a very high degree of sequence similarity with a cadmium-binding Mt from *Helix pomatia*, a species that is closely related to *Arianta arbustorum*. Moreover, Mta and Mtb, as expected, also exhibit structural similarities with Mts from other molluscan species, such as mussels and oysters. It is suggested that Mta and Mtb represent two allelic isoforms, reflecting the genetic polymorphism of Mt in *Arianta arbustorum*.

INTRODUCTION

Since their discovery in the 1950s [1], metallothioneins (Mts) have been, and still are, a matter for intensive research [2]. What makes these proteins so interesting is the fact that they possess a variety of biochemical and structural features, which are unique among all proteins known so far. Moreover Mts seem to exhibit a large range of different functions, and new ones are still being found [3].

The best-studied Mts are those from mammalian species, including the horse [4], the cow [5], the mouse [6], the rat [7], the rabbit [8,9] and, of course, the human [10]. Primary structures of non-mammalian Mts have been elucidated via cDNA for fish [11] and birds [12]. Concerning invertebrates, primary structures have been reported for Mts from crabs [13,14], insects [15], molluscs [16–18] and, very recently, from protozoans [19]. Among non-mammalian Mts, the best-studied proteins are those from some invertebrates, such as the sea urchin [20] and the crab [13], for which amino acid sequences and tertiary structure investigations [21,22] have been reported.

From all these studies it appeared that Mts are low-molecular-mass cysteine-rich proteins, which normally lack aromatic amino acids. They exhibit a characteristic amino acid sequence with highly conserved positions for cysteine residues [23]. By means of the sulphur atoms of the cysteines, the proteins bind specifically some 'soft' metal ions such as Cd^{2+}, Zn^{2+} and Cu^{+}, forming characteristic metal–thiolate clusters [24]. In most species, Mts occur in several isoforms which may differ from each other by only a few amino acid positions [2]. As shown by two-dimensional NMR and X-ray crystallography studies [25,26], the spatial structure of the Mt molecule from mammals is dominated by two metal-binding domains: the C-terminal α-domain, which binds four metal ions, and the N-terminal β-domain, binding three metal ions [2]. In crab Mt, the situation is slightly different, since in this case both domains bind three metal ions [21].

The function of Mts is still a matter of discussion. It appears that Mt synthesis is inducible by some 'soft' metal ions [13]. Moreover, Mts seem to confer resistance to cells and organisms against intoxication with cadmium or copper [27,28]. Because of these findings, it has been argued that Mts may primarily be involved in metal detoxification. An important role of Mts has also been shown in trace element homoeostasis and regulation [29,30]. In decapod crustaceans, for instance, Mts have been shown to play an important role in connection with copper supply during moulting [31]. In this context it has been suggested that Mts may simultaneously be responsible for the detoxification of non-essential trace elements, such as cadmium, on the one hand, and the regulation of essential trace elements, such as copper and zinc, on the other [23,32]. Apart from metal detoxification and regulation, additional functions have been attributed to Mts, such as the intracellular scavenging of free radicals in protecting cells against oxidative stress [33,34], and zinc-mediated gene regulation [35].

In the present study, two Mt isoforms were purified and sequenced from the terrestrial gastropod, *Arianta arbustorum*. The aim was to investigate by how much the primary structure

Abbreviations used: Mt(s), metallothionein(s); TFA, trifluoroacetic acid; ES-MS, electrospray mass spectrometry; CID, collision-induced dissociation; MS/MS, tandem mass specrometry.

§To whom correspondence should be addressed.

The novel protein sequence data published here have been submitted to the Protein Identification Resource (PIR) sequence database.

of Mt isoforms would differ among closely related gastropod species on the one hand, and within one single species on the other. Lastly, the present study was also undertaken with regard to the potential use of terrestrial invertebrate Mts as biomarkers for environmental pollution.

MATERIALS AND METHODS

Chemicals, reagents and enzymes

DEAE-cellulose was from Whatman BioSystems Ltd. (Maidstone, U.K.). Sephacryl S-100 was from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). Sephadex G-25, PMSF and Trizma base were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acetonitrile for HPLC (gradient-grade), trifluoroacetic acid (TFA), and 2-mercaptoethanol were from Merck (Darmstadt, Germany).

Trypsin (EC 3.4.21.4) from bovine pancreas, endoproteinase Asp-N (EC 3.4.24.33) from a *Pseudomonas fragi* mutant, and endoproteinase Glu-C (EC 3.4.21.19) from *Staphylococcus aureus* V8 were all sequencing grade, from Boehringer (Mannheim, Germany).

All other chemicals and reagents were from Merck (Darmstadt, Germany) or from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Animals and cadmium exposure

Arianta arbustorum (40 specimens; 2.0–2.5 g individual fresh wt.) were collected in summer 1992 from a garden near Innsbruck (Tyrol, Austria). The animals were transferred to the laboratory and reared in plastic boxes with moistened garden soil under constant conditions (18 °C, 12 h light: 12 h dark) over 2 months. The snails were fed on cadmium-enriched lettuce ($CdCl_2$, approx. 150 μg/g dry weight) twice a week, as described by Dallinger et al. [17].

Mt purification

At the end of the feeding period, all snails were killed and dissected. Midgut glands of five individuals were pooled for each sample, yielding a mean tissue fresh weight of 1.5 g for each of eight aliquots, and stored for several days at −70 °C.

After thawing, each sample aliquot was processed in the following way: tissues were homogenized in 2 vol. of 25 mM Tris/HCl buffer (containing 100 mM NaCl, 20 mM 2-mercaptoethanol and 0.1 mM PMSF), pH 7.5, and centrifuged for 1 h at 27000 *g*. The pellet was discarded and 2 g of DEAE-cellulose was added to the supernatant. After thoroughly stirring for several minutes, the DEAE-cellulose was sedimented by centrifugation at 20000 *g* over 5 min. The resulting supernatant was filtered (Sartorius Minisart, 0.2 μm pore size) and applied to a gel-permeation chromatography column (15 mm × 30 cm) packed with Sephacryl S-100. Column calibration was achieved by applying a mixture of the following substances: Blue Dextran (2000 kDa), chicken egg albumin (45 kDa), myoglobin (18.5 kDa) and vitamin B_{12} (1.35 kDa). Elution was performed with a 25 mM Tris/HCl buffer (containing 10 mM 2-mercaptoethanol), pH 7.5, at a flow rate of 12 ml/min, using an HPLC pump (Waters, model 501). In fractions of 4 ml, absorbance at 280 and 254 nm and cadmium concentrations were measured. Cadmium-containing fractions were pooled and concentrated by ultrafiltration (Amicon YM2, 1 kDa cut-off) to one-tenth of the original volume. Aliquots of these concentrates (500 μl each) were fractionated on an HPLC system (Waters, model 501) equipped with a multiwavelength detector (Waters, model 490E), using a 3.9 mm × 300 mm μBondapack C_{18} reversed-phase column (10 μm particle size, 12.5 nm pore size). Elution was performed over 25 min at a flow rate of 1 ml/min, using solvent A (25 mM Tris/HCl containing 5 mM sodium azide, pH 7.5) in a gradient of 0–40% solvent B (25 mM Tris/HCl, pH 7.5, containing 5 mM sodium azide and 60% acetonitrile). In fractions of 1 ml, absorption at 254 and 280 nm was recorded, and cadmium concentrations were measured. From each of two distinct cadmium-containing peaks, (Cd)-Mt fractions were pooled and stored for several days at −70 °C. As shown below, each of these pools represented a separate Mt isoform, called Mta and Mtb.

Metal analyses

Cadmium concentrations in fractions derived from gel chromatography and from reversed-phase HPLC were measured by means of atomic absorption spectrophotometry (Perkin–Elmer, model 2380). Solutions were aspirated directly into the flame of the instrument, using deuterium background correction.

Recovery of apo-Mt and cysteine modification

Apo-proteins of both Mt isoforms were prepared by acidifying the respective (Cd)-Mt fractions from reversed-phase HPLC with 1% TFA, and by applying the proteins to a Sephadex G-25 column (15 mm × 20 cm). They were then eluted at a flow rate of 3 ml/min with 0.1% TFA. The resulting protein fractions were desiccated in a vacuum concentrator (Savant Instruments, Speed-Vac System I). For amino acid sequencing, the apo-Mts were modified by S-methylation, using the method described by Hunziker [36]. The pure S-methylated proteins were desalted by elution on a Sephadex G-25 column with 0.1% TFA, as described above.

Endoproteinase digestion and peptide mapping

The two S-methylated isoforms were each digested with three endoproteinases: trypsin, endoproteinase Glu-C and endoproteinase Asp-N, following the instructions of the supplier. Resultant peptides of both proteins were recovered by reversed-phase HPLC (Waters model 501), using a 3.9 mm × 300 mm μBondapack C_{18} reversed-phase column (10 μm particle size, 12.5 nm pore size). The injected volume was 500 μl. Elution of Glu-C and Asp-N peptides was performed over 55 min at a flow rate of 0.6 ml/min, using solvent A (0.1% TFA) in a gradient of 0–60% solvent B (solvent A containing 60% acetonitrile). Recovery of tryptic peptides was achieved by eluting the sample with the same solvents as described above, using a gradient of 0–25% solvent B over 30 min, and 25–45% solvent B over 55 min.

Amino acid analysis

Proteolytic peptides from endoproteinase digests of both Mt isoforms were analysed for amino acid composition with a 420A/H amino acid analyser (Applied Biosystems, U.S.A.), according to the manufacturer's instructions.

Sequence analysis

Amino acid sequences of proteolytic peptides from both Mt isoforms were determined by automated Edman degradation, using a model 477A sequencer (Applied Biosystems, U.S.A.), equipped with an on-line phenylthiohydantoin-amino-acid analyser (model 120A, Applied Biosystems, U.S.A.). All reagents

used were from Applied Biosystems and were prepared according to the instructions of the manufacturer.

MS analysis

The relative molecular masses of most of the proteolytic fragments of both Mta and Mtb were determined by electrospray MS (ES-MS) using an API III triple-quadrupole instrument (Sciex, Canada). The collected peptides were freeze-dried and redissolved 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.

Some of the peptides were completely characterized by collision-induced dissociation (CID) tandem mass spectrometry (MS/MS) using argon as the collision gas at a thickness of approximately 4×10^{14} molecules/cm^2.

Strategy of sequence determination

The strategy of sequence determination of Mta and Mtb from *Arianta arbustorum* was based on their expected homology with the already-known Mt from *Helix pomatia* [17] and on the fact, that Mtb was available in higher concentrations than Mta. Therefore, the sequence of Mtb was determined first, using automated Edman degradation and ES-MS as well as CID-MS/MS for the N-terminal tryptic peptide, which was blocked for Edman degradation due to acetylation of its N-terminal serine. After proper alignment of sequences from proteolytic Mtb fragments, the sequence of Mta was derived in a second step, by comparing data from combined ES-MS and amino acid analyses of Mta peptides with those of Mtb. Moreover, one single peptide which differed from the reported Mtb sequence by one amino acid exchange, was sequenced by CID-MS/MS.

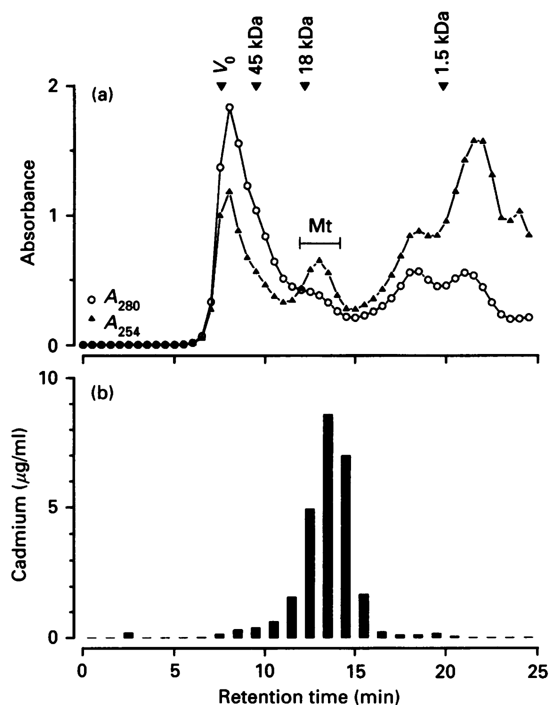

Figure 1 Elution profile of midgut gland supernatant from cadmium-exposed *Arianta arbustorum* after gel-permeation chromatography (Sephacryl S-100), showing absorbance at 280 and 254 nm (a), and cadmium concentration (b)

(**a**) Retention times (arrowheads) and molecular masses of marker substances for column calibration are also given. The range of cadmium-containing Mt fractions (Mt) pooled for further purification is marked by the horizontal bar in (**a**).

RESULTS AND DISCUSSION

Purification of Mt isoforms and peptide mapping

After gel-permeation chromatography, all the cadmium was associated with fractions of an apparent molecular mass of 15 kDa (Figure 1). These fractions were also characterized by an increased absorbance at 254 nm (Figure 1a). Apart from cadmium, they also contained some copper and zinc (results not shown). After pooling and concentration by ultrafiltration, these cadmium-containing components were applied to reversed-phase HPLC, yielding an elution profile with two major peaks, absorbing at 254 nm, called Mta and Mtb (Figure 2a). Most of the cadmium eluted with these two peaks (Figure 2b). On the basis of their amino acid compositions, components from both peaks could be identified as Mts. Besides the two main Mt peaks (Mta and Mtb), the elution profile (Figure 2) indicated the presence of additional, cadmium-containing components, the absorbance of which was considerably less than that of the two main peaks. Although all these small peaks probably also represented Mt isoforms or variants, only the fractions of Mta and Mtb were collected for further sequence analysis.

Because of the inducibility of Mta and Mtb by cadmium, and due to their cadmium loading, it would be tempting to think of a role for these proteins in connection with the detoxification of cadmium. Some species of terrestrial gastropods have indeed been shown to be highly tolerant of elevated concentrations of cadmium in their diet [37], this increased tolerance being based on the metal-induced synthesis of cadmium-binding Mts [38].

In the present study, reversed-phase HPLC of proteolytic peptides showed similar elution patterns for Mta and Mtb. Due to the elevated lysine content of both isoforms, the highest number of proteolytic peptides was recovered after trypsin digestion (Figure 3), followed by digestions with the endoproteinases Glu-C and Asp-N, with three proteolytic peptides each (results not shown). As seen in Figure 3, the elution profile of tryptic peptides yielded six (Mta) or seven (Mtb) marked peaks, containing eight different peptide fragments. The peptide T3 of Mtb (Figure 3a) differed from the analogous peptide of Mta by one amino acid exchange. Because of this difference, the respective peptide of Mta (T3′) was found within one single peak together with peptide T2′ (Figure 3b).

Amino acid sequence and comparison of Mta and Mtb with invertebrate and vertebrate Mts

For most of the proteolytic Mtb peptides, sequences were derived from Edman degradation (Table 1). Only in the case of the short Asp-N peptide (A1), which was blocked for Edman degradation by acetylation of its N-terminal serine, was the sequence determined by CID-MS/MS. Moreover, the relative masses of most of the Mtb peptides (except for T1) were determined by means of ES-MS (Table 1), and were found to correspond well with the theoretical relative masses derived from their known sequences.

Because of the expectedly high sequence similarity between Mta and Mtb, most of the proteolytic Mta peptides were not subjected to sequence analysis (except for T2′); instead of this, numbers of amino acid residues found by amino acid analysis were compared with those found in analogous tryptic peptides of Mtb (compare Tables 1 and 2). Moreover, relative masses of

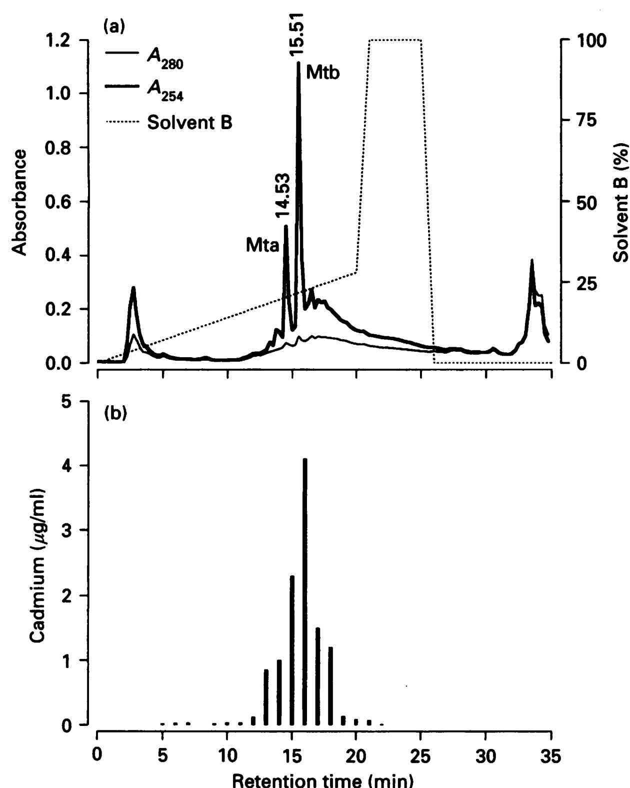

Figure 2 Reversed-phase HPLC of cadmium-Mt, derived from pooled gel chromatography fractions (see Figure 1), showing absorbance at 280 and 254 nm in a gradient of solvent B (a), and cadmium concentration (b)

(a) Also shown are retention times of the the two main Mt isoforms (Mta and Mtb).

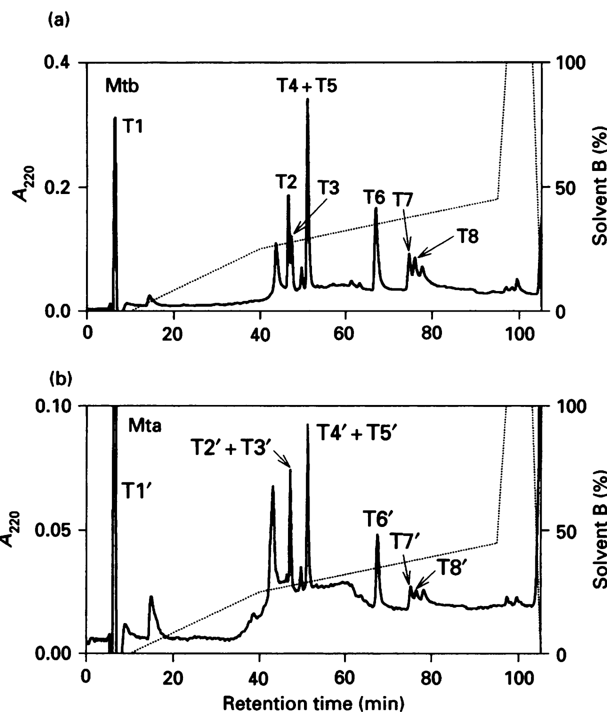

Figure 3 Peptide map of tryptic Mtb (a) and Mta (b) peptides after reversed-phase HPLC, showing absorbance at 220 nm in a gradient of solvent B

Tryptic peptides of Mtb (T1–T8) and Mta (T1′–T8′) are numbered in increasing order of retention times.

tryptic Mta peptides were determined by MS, and compared with theoretical relative masses which would have to be expected due to their homologies with corresponding peptides of Mtb (Table 2). In fact, most of these relative masses were found to be consistent with the theoretically expected values. Only the peptide T2′ showed a relative mass which was significantly higher than that of the corresponding peptide T2 of Mtb. The relative mass difference between the two peptides was found to be 57. As shown by sequence determination, this difference was due to an amino acid exchange at position 2 of the peptide, in which a glycine residue in T2 of Mtb was replaced by an asparagine residue in T2′ of Mta (Figure 4).

The alignment of Mtb and Mta (Figure 4) shows that the two Mt isoforms of *Arianta arbustorum* are structurally identical, differing only by one amino acid exchange at position 60 of the sequence. Both isoforms consist of 66 amino acids, 18 of which are cysteine residues. Apart from this, the N-terminal amino acid of both isoforms consists of an acetylated serine residue. The calculated molecular masses (without the N-terminal acetylation) are 6.43 kDa for Mtb and 6.49 kDa for Mta.

The primary structures of Mtb and Mta of *Arianta arbustorum* reveal that both protein isoforms belong to class-I Mts (Figure 4). As proposed by Kojima [39], class-I Mts are defined as polypeptides with locations of cysteine residues closely related to those in equine renal Mt. The 18 cysteine residues of Mtb and Mta are characteristically arranged in seven Cys-Xaa-Cys motifs (Figure 5). It has been stated that the arrangement of cysteine residues in Cys-Cys, Cys-Xaa-Cys, or Cys-Xaa-Yaa-Cys motifs (in which Xaa and Yaa denote any amino acid except cysteine) within the polypeptide chain is the basis for the high affinity of Mts for soft-metal ions [2].

The amino acid sequences of Mtb and Mta of *Arianta arbustorum* show a high degree of similarity with a recently characterized cadmium-binding Mt of the Roman snail, *Helix pomatia*, and with Mts of other mullusc species (Figure 5) [17]. As in the Roman snail, a histone-like N-terminus is also found in Mtb and Mta from *Arianta arbustorum*, although in the two latter polypeptides the degree of similarity with histones is somewhat less than that observed for the Mt of *Helix pomatia*, due to replacements of two amino acid residues at positions 7 and 8 of the protein chain (Figure 5). The significance of this histone-like motif in snail Mts remains obscure.

A sequence comparison on the basis of FASTA scores reveals, apart from an expectedly high homology between snail and mollusc Mts, that the degree of identity between snail and vertebrate Mts is higher than that observed between snail Mts and those from other invertebrates (Table 3). This high degree of similarity with vertebrate Mts also holds for other known molluscan Mts, such as those of oyster and mussel [18]. On the basis of these close relationships in their primary structure, molluscan Mts can be placed, together with vertebrate Mts, within class I of the Mt family. In contrast, Mts from some other invertebrates, including the fruit fly, the nematode, and the sea urchin, belong to class-II Mts [2]. In spite of this divergence, it has been shown that snail Mts share several structural elements, such as repeated Cys-Xaa-Cys motifs, and a small conserved motif in the central region of the polypeptide chain, with most Mts of other invertebrates [17,40].

Table 1 Sequences and relative masses of proteolytic peptides of Mtb, recovered after digestion with trypsin (T1–T8), endoproteinase Glu-C (G1–G3), and endoproteinase Asp-N (A1–A3)

Sequences and relative masses (expected: theoretically expected from sequence determination or from comparison with homologous peptides from Cd-Mt of the closely related species, *Helix pomatia* [17]; found: found by MS of proteolytic peptides of Mtb. For most peptides, sequence determination was carried out by automated Edman degradation. Only the peptide A1 was sequenced by CID-MS/MS. The blocked N-terminal peptides T8 and G3 were not sequenced. Abbreviations: n.d., not determined; Ac, N-terminal acetylation.

		Relative masses	
Peptide	Sequence found	Expected	Found
T1	CGK	321.2	n.d.
T2	CGSSCSCK	816.3	816.0
T3	ECTGAASCK	897.4	896.7
T4	TCNCTSDGCK	1073.4	1072.0
T5	CGKECTGAASCK	1199.5	1198.6
T6	CQCGEGCACASCK	1332.5	1331.5
T7	GKGDLCTAACKNEPCQCGSK	2068.9	2067.6
T8	n.d. (blocked N-terminal fragment)	†2383.1	†2383.0
G1	CTGAASCKCGSSCSCK	1565.6	1564.6
G2	GCACASCKTCNCTSDGCKCGKE	2269.9	2269.8
G3	n.d. (blocked N-terminal fragment)	†2931.3	†2932.6
A1	*Ac-SGKGKG	†575.3	†572.1
A2	DGCKCGKECTGAASCKCGSSCSCK	2414.0	2414.0
A3	DLCTAACKNEPCQCGSKCQCGEGCACASCKTCNCT	3777.5	3779.2

* Determined by CID-MS/MS.
† Including the N-terminal acetyl group

Table 2 Amino acid analyses (numbers of residues found/peptide) of tryptic fragments of Mta (T1′–T8′), as well as relative masses

The theoretically expected relative masses (Expected) were derived from sequences of analogous tryptic Mtb peptides (compare Table 1). Values listed under 'Found' were determined by MS. Also shown is the sequence of the only Mta peptide, which differs from the respective Mtb peptide by its relative mass, due to one amino acid exchange (N for G) (see Table 1). Abbreviation: n.d., not determined.

		Relative masses	
Peptide	Amino acid analysis (sequence)	Expected	Found
T1′	Cys/Gly/Lys	321.2	n.d.
T2′	n.d. *(CNSSCSCK)	816.3	873.2
T3′	n.d.	897.4	896.3
T4′	Mixture of T4′ and T5′:	1073.4	1072.5
T5′	$Cys_6/Asx_2/Glx/Ser_2/Gly_3/Thr_3/Ala_2/Lys_3$	1199.5	1198.7
T6′	$Cys_5/Glx_2/Ser/Gly_2/Ala_2/Lys$	1332.5	1331.6
T7′	$Cys_4/Asx_2/Glx_2/Ser/Gly_3/Thr/Ala_2/Pro/Leu/Lys_3$	2068.9	2068.8
T8′	n.d.	†2383.1	†2383.2

* Determined by CID-MS/MS.
† Including the N-terminal acetyl group

So far, insufficient information is available concerning the tertiary structure of gastropod Mts. However, it has been demonstrated that even sea urchin Mt, which deviates considerably from mammalian Mts in its primary structure, exhibits a spatial organization of metal–thiolate clusters similar to that observed in mammalian Mts [22]. Thus it may be speculated that this is also true for gastropod Mts which resemble mammalian Mts more closely than sea urchin Mts do.

The present study revealed, that Mta and Mtb of *Arianta arbustorum* represent two very closely related isoforms, apparently reflecting the polymorphism of Mts in this species.

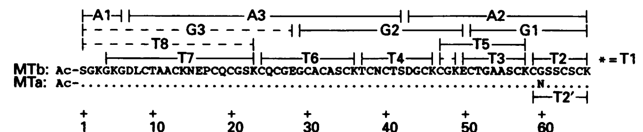

Figure 4 Overlaps of proteolytic peptides (horizontal bars) of Mtb, recovered after digestion with endoproteinase Asp-N (A1–A3), endoproteinase Glu-C (G1–G3) and trypsin (T1–T8), and of a tryptic peptide of Mta (T2′ only), as well as sequences and alignments of Mtb and Mta (including N-terminal acetylation, Ac-)

Sequenced peptides are marked by solid-line bars, non-sequenced peptides (for which only relative masses were determined) are marked by broken-line bars. All peptide sequences were derived from automated Edman degradation, except for peptides A1 and T2′, which were sequenced by CID-MS/MS.

Throughout the animal kingdom polymorphism of Mts is a frequently observed phenomenon [2]. So far, the highest numbers of Mt isoforms from one single species have been reported for the human [3] and the mussel, *Mytilus edulis* [18]. However, according to the definition of multiple protein forms by the IUPAC-IUB Commission on Biochemical Nomenclature [41], and in agreement with the nomenclature of Mts, the term 'isometallothionein' should only be adopted for Mt forms arising from genetically determined differences in their primary structure [39]. Mt variants that are post-translationally modified due to, for instance, acetylation of the N-terminal amino acid residue, or processing of the N-terminal methionine, should not be called isoforms.

It has been argued that even small differences in the primary structure of Mt isoforms may be responsible for differences in metal affinities [42]. Most authors agree, however, that differences in the primary structure of Mt isoforms offer only one of several explanations for the multi-functionality of these proteins. In the present study, the differences in the primary structure between

Species: Mt	Sequence
Helix pomatia: Cd-Mt[17]	Ac-SGKGKGEKCTSACRSEPCQCGSKCQC GEGCTCAACK TCNCTSDG CKCGKECTGPDSCKCNSSCSCK
Arianta arbustorum: Mta[*]	Ac-SGKGKGDLCTAACKNEPCQCGSKCQC GEGCACASCK TCNCTSDG CKCGKECTGAASCKCNSSCSCK
Arianta arbustorum: Mtb[*]	Ac-SGKGKGDLCTAACKNEPCQCGSKCQC GEGCACASCK TCNCTSDG CKCGKECTGAASCKCGSSCSCK
Crass. virginica: Mt[55]	MSDPCNCIETGTCACSDSCPATGCKCGPGCKC GDDCKCAGCKVKCSCTSEGGCKCGEKCTGPATCKCGSGCSCKK
Mytilus edulis: Mt 10-I[18]	PAPCNCIETNVCICDTGCSGEGCRCGDACKCSGADCKCSGCKVVCKC SGRCECGKGCTGPSTCKCAPGCSCK
Mytilus edulis: Mt 20-Ia[18]	PGPCNCIETNVCICGTGCSGKCCRCGDACKCASG CGCSGCKVVCKC SGTCKCGCDCTGPTNCKCESGCSCK

Figure 5 Species names, Mt specification (superscripts indicate references; [*] indicates this study) and alignment of known molluscan Mts, performed by means of the PILEUP program (GCG package), and edited by eye, using the program ESEE version 1.09 [49]

Identical amino acid positions are marked by vertical dashes. Abbreviations: *Crass.*, *Crassostrea*; Ac-, N-terminal acetylation.

Table 3 Comparison of Mta and Mtb from *Arianta arbustorum* with invertebrate and vertebrate Mts, by screening of the Protein Identification Resource data base (PIR) (release 41), with the FASTA Sequence Comparison Program [43] of the GCG package, version 8.0

Abbreviations: *Crass.*, *Crassostrea*; *Caenorh.*, *Caenorhabditis*; *Strong.*, *Strongylocentrotus*; *Hom.*, *Homarus*; *Oncorh.*, *Oncorhynchus*.

			Optimized FASTA score		Identity (%)		Overlaps (number of residues)	
Systematic group: species	Mt isoform	Reference	Mta	Mtb	Mta	Mtb	Mta	Mtb
Invertebrates								
Mollusca:								
Helix pomatia	Mt	[17]	371	376	84.8	86.4	66	66
Crass. virginica	Mt	[44]	265	270	56.7	58.3	60	60
Mytilus edulis	10-II	[18]	245	246	49.2	49.2	61	61
Nematoda:								
Caenorh. elegans	MtI	[40]	138	138	36.8	36.8	57	57
Echinodermata:								
Strong. purpuratus	MtA	[20]	126	126	38.9	38.9	36	36
Crustacea:								
Scylla serrata	Mt1	[13]	134	134	35.1	35.1	57	57
Vertebrates								
Fish:								
Esox lucius	Mt	[45]	156	155	41.1	41.1	56	56
Birds:								
Gallus gallus	Mt	[46]	135	133	34.5	40.0	58	60
Mammals:								
Equus caballus	Mt1A	[47]	137	138	32.0	32.0	50	50
Homo sapiens	MtIII	[48]	147	148	39.2	39.2	51	51

the two isoforms are so slight that they would hardly be able to perform different functions. In fact, it cannot be excluded that Mtb and Mta from *Arianta arbustorum* are two allelic isoforms. The reason for this assumption is based on the fact that, in order to get sufficient amounts of tissue for the isolation procedure, a number of individuals had to be pooled. The same explanation was also given by Mackay et al. [18] for the presence of different, but closely related, Mt isoforms in the marine mussel, *Mytilus edulis*. Such a high degree of allelic polymorphism might be due to the increased heterozygosity in invertebrates, as compared with vertebrates [18].

We thank Dr. R. Schneider (Institut für Biochemie der Universität Innsbruck) for helpful discussions and for assistance in using the protein sequence database. W. Wieser (Institut für Zoologie der Universität Innsbruck) was instrumental in reviewing the manuscript. This work has been supported by the Austrian Fonds zur Förderung der wissenschaftlichen Forschung, project no. P9770. B.B. was supported by a grant from the Österreichische Akademie der Wissenschaften (APART – Austrian Programme for Advanced Research and Technology).

REFERENCES

1 Margoshes, M. and Vallee, B. L. (1957) J. Am. Chem. Soc. **79**, 4813–4818
2 Kägi, J. H. R. (1993) in Metallothionein III (Suzuki, K. T., Imura, N. and Kimura, M., eds.), pp. 29–55, Birkhäuser Verlag, Basel
3 Vallee, B. L. and Maret, W. (1993) in Metallothionein III (Suzuki, K. T., Imura, N. and Kimura, M., eds.), pp. 1–27, Birkhäuser Verlag, Basel
4 Kägi, J. H. R. and Kojima, Y. (1987) Experientia **52** (Suppl.), 25–61
5 Münger, K., German, U. A., Beltramini, M. et al. (1985) J. Biol. Chem. **260**, 10032–10038
6 Huang, I., Kimura, M., Hata, A., Tsundoo, H. and Yoshida, A. (1981) J. Biochem. (Tokyo) **89**, 1839–1845
7 Winge, D. R., Nielson, K. B., Zeikus, R. D. and Gray, W. R. (1984) J. Biol. Chem. **259**, 11419–11425
8 Wagner, G., Neuhaus, D., Wörgötter, E., Vasak, M., Kägi, J. H. R. and Wüthrich, K. (1986) Eur. J. Biochem. **157**, 275–289
9 Hunziker, P. E., Kaur, P., Wan M. and Kaenzig, A. (1995) Biochem. J. **306**, 265–270
10 Hunziker, P. E. and Kägi, J. H. R. (1985) Biochem. J. **231**, 375–382
11 Bonham, K., Zafarullah, M. and Gedamu, L. (1987) J. Cell Biol. **6**, 519–527
12 Wei, D. and Andrews, G. K. (1988) Nucleic Acids Res. **16**, 537–553

13 Lerch, K., Ammer, D. and Olfafson, R. W. (1982) J. Biol. Chem. **257**, 2420–2426
14 Pedersen, K. L., Pedersen S. N., Højrup, P., Andersen, J. S., Roepstorff, P., Knudsen, J. and Depledge, M. H. (1994) Biochem. J. **297**, 609–614
15 Lastowski-Perry, D., Otto, E. and Maroni, G. (1985) J. Biol. Chem. **260**, 1527–1530
16 Roesijadi, G., Kielland, S. and Klerks, P. (1989) Arch. Biochem. Biophys. **273**, 403–413
17 Dallinger, R., Berger, B., Hunziker, P. E., Birchler, N., Hauer, C. R. and Kägi, J. H. R. (1993) Eur. J. Biochem. **216**, 739–746
18 Mackay, E. A., Overnell, J., Dunbar, B., Davidson I., Hunziker, P. E., Kägi, J. H. R. and Fothergill, J. E. (1993) Eur. J. Biochem. **218**, 183–194
19 Piccinni, E., Staudenmann, W., Albergoni, V., De Gabrieli, R. and James, P. (1995) Eur. J. Biochem. **226**, 853–859
20 Nemer, M., Wilkinson, D. G., Travaglini, E. C., Sternberg, E. J. and Butt, T. R. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 4992–4994
21 Otvos, J. D., Olfafson, R. W. and Armitage, I. M. (1982) J. Biol. Chem. **257**, 2427–2431
22 Wang, Y., Mackay, E. A., Kurasaki, M., Zerbe, O., Hess, D., Vasak, M. and Kägi, J. H. R. (1994) in 15th Conference of the European Society for Comparative Physiology and Biochemistry, Genova, September 1994, p. 90
23 Hamer, D. H. (1986) Annu. Rev. Biochem. **55**, 913–951
24 Kägi, J. H. R. (1991) Methods Enzymol. **205**, 613–626
25 Wüthrich, K. (1991) Methods Enzymol. **205**, 502–520
26 Robbins, A. H., McRee, D. E., Williamson, M. et al. (1991) J. Mol. Biol. **221**, 1269–1293
27 Hamer, D. H. and Thiele, D. J. (1985) Science **228**, 685–690
28 Maroni, G., Wise, J., Young, J. E. and Otto, E. (1987) Genetics **117**, 739–744
29 Richards, M. P. (1989) J. Nutr. **119**, 1062–1070
30 Bremner, I. (1991) Methods Enzymol. **205**, 25–35
31 Engel, D. W. and Brouwer, M. (1987) Biol. Bull. **173**, 239–251
32 Engel, D. W. and Brouwer, M. (1989) Adv. Comp. Environ. Physiol. **5**, 53–75
33 Abel, J. and de Ruiter, N. (1989) Toxicol. Lett. **47**, 191–196
34 Sato, M., Sasaki, M. and Hojo, H. (1993) in Metallothionein III (Suzuki, K. T., Imura, N. and Kimura, M., eds.), pp. 125–140, Birkhäuser Verlag, Basel
35 Zeng, J., Heuchel, R., Schaffner, W. and Kägi, J. H. R. (1991) FEBS Lett. **279**, 310–312
36 Hunziker, P. E. (1991) Methods Enzymol. **205**, 399–400
37 Berger, B., Dallinger, R., Felder, E. and Moser, J. (1993) in Ecotoxicology of Metals in Invertebrates (Dallinger, R. and Rainbow, P. S., eds.), pp. 291–313, Lewis Publishers, Boca Raton, Florida
38 Dallinger, R. (1993) in Ecotoxicology of Metals in Invertebrates (Dallinger, R. and Rainbow, P. S., eds.), pp. 245–289, Lewis Publishers, Boca Raton, Florida
39 Kojima, Y. (1991) Methods Enzymol. **205**, 8–10
40 Imagawa, M., Onozawa, T., Okumura, K., Osada, S., Nishimura, T. and Kondo, M. (1990) Biochem. J. **268**, 237–240
41 IUPAC-IUB Commission on Biochemical Nomenclature (1977) J. Biol. Chem. **252**, 5939–5941
42 Winge, D. R. and Miklossy, K. A. (1982) J. Biol. Chem. **257**, 3471–3476
43 Pearson, W. R. and Lipman, D. J. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 2444–2448
44 Unger, M. E., Chen, T. T., Murphy, C. M., Vestling, M. M., Fenselau, C. and Roesijadi, G. (1991) Biochim. Biophys. Acta **1074**, 371–377
45 Kille, P., Kay, J. and Sweeney, G. E. (1993) Biochim. Biophys. Acta **1216**, 55–64
46 Fernando, L. P. and Andrews, G. K. (1989) Gene **81**, 177–183
47 Kojima, Y. and Kägi, J. H. R. (1978) Trends Biochem. Sci. **3**, 90–93
48 Palmiter, R. D., Findley, S. D., Whitmore, T. E. and Durnam, D. M. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 6333–6337
49 Cabot, E. L. and Beckenbach, A. T. (1989) Comput. Appl. Biosci. **5**, 233–234

Received 7 February 1995/20 June 1995; accepted 23 June 1995