

Purification and characterization of a cadmium-induced metallothionein from the shore crab *Carcinus maenas* (L.)

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Two metallothionein variants were purified from the midgut gland of crabs (*Carcinus maenas*) exposed to a high cadmium concentration (2 p.p.m.). One of the variants was purified from crabs exposed to a low cadmium concentration (0.5 p.p.m.). The purification method involved acetone precipitation, gel filtration and reversed-phase h.p.l.c. The complete amino acid sequences of both variants have been elucidated by m.s. and automated sequence analysis on *S*-methylated proteins or fragments produced by cleavage of the *S*-methylated proteins with *Staphylococcus aureus* proteinase. The two variants from crabs exposed to the high cadmium concentration differed only by a single

residue of methionine at the N-terminus. The single variant isolated from crabs exposed to the low cadmium concentration was the one without the N-terminal methionine, indicating that high cadmium concentrations either inhibit the processing enzymes and/or that the processing enzymes cannot keep pace with the increased metallothionein synthesis when cadmium availability is high. Cadmium-induced metallothionein from *C. maenas* shows a high degree of structural similarity to metallothioneins from the decapod crustaceans *Scylla serrata* and *Homarus americanus*.

INTRODUCTION

Metallothioneins (MTs) are low-molecular-mass cysteine-rich proteins capable of binding heavy metals (Hamer, 1986). They have been found in a variety of prokaryotic and eukaryotic organisms including vertebrates, invertebrates, plants and microorganisms (Hamer, 1986; Kägi and Kojima, 1987). MT was first isolated in 1957 as a Cd²⁺ binding protein from equine kidney cortex (Margoshes and Vallee, 1957). Subsequent studies have indicated that MTs bind a wide range of heavy metals and it is now thought that they play a central role in the handling and detoxification of several heavy metals (Templeton and Cherian, 1991). MTs may also play a key role in regulation of essential metals such as Zn²⁺ and Cu²⁺. Currently, the potential for using metallothionein as an ecotoxicological biomarker of heavy-metal pollution is being explored. It is in this latter context that the present study was undertaken.

The N- and C-terminal regions of the mammalian MT molecule contain two separate metal–thiolate clusters, one which binds four metal ions (cluster A) and the other, three (cluster B) (Kägi and Kojima, 1987). MTs from the crab *Scylla serrata* also contain two separate clusters, but these exist as a pair of identical three-metal ion clusters analogous to the mammalian cluster B (Otvos et al., 1982). In mammals, cluster A binds Cd²⁺ and Zn²⁺ more avidly than does cluster B (Bernhard et al., 1986, 1987), and the reverse is the case for binding of Cu²⁺ (Briggs and Armitage, 1982). Thus crustacean MTs contain only the low-affinity cluster, and consequently their overall affinity for Cd²⁺ and Zn²⁺ at least might be expected to be lower than that of vertebrate MTs. This might also explain the higher susceptibility of crustacean MTs to oxidation (Wong and Rainbow, 1986; Overnell, 1986).

The first invertebrate MT-like proteins were purified from the tissues of marine molluscs (Casterline and Yip, 1975; Noël-Lambot, 1976) and decapod crustaceans (Jennings et al., 1979). However, except for reports on the primary and secondary

structure of MT from the crab *S. serrata* (Lerch et al., 1982; Otvos et al., 1982) and a partial primary structure for MT from the lobster *Homarus americanus* (Brouwer et al., 1989), progress in understanding the biochemical, physiological and toxicological significance of crustacean MTs has been impeded by the inability to purify and characterize these metal-binding proteins.

The majority of MTs exhibit genetic polymorphism and two main isomers (MTI and MTII), which are coded for by non-allelic genes, are usually expressed. The two MT-isomers (particularly MTI) often exist in several subforms, i.e. at least five hepatic isoforms of MTI have been reported in humans (Kägi and Kojima, 1987). Among crustaceans, two isomers have been sequenced from the crab *S. serrata* (Lerch et al., 1982), and MT polymorphisms have been postulated for several other crustacean species, e.g. *Callinectes sapidus* (Schlenk and Brouwer, 1991) and *H. americanus* (Brouwer and Brouwer-Hoexum, 1991). Similarly in fish, most species have two MT isomers (Roesijadi, 1992), but it appears that there is only one MT gene in turbot (George et al., 1992) and in winter flounder (Chan et al., 1989). An analogous situation is seen in birds where two isomers have been reported in quail and pigeon (Yamamura and Suzuki, 1984; Lin et al., 1989) but only one in chicken and duck (McCormick et al., 1988; Lin et al., 1990).

In the present study, a rapid and simple method for the purification of MTs from invertebrates has been developed which involves acetone precipitation, gel filtration and reversed-phase h.p.l.c. This technique has been used to purify a major MT isomer from the midgut gland of *Carcinus maenas* exposed to Cd²⁺ (2 p.p.m.). The purified MT was sequenced and the primary structure compared with other invertebrate MTs.

MATERIALS AND METHODS

Enzymes and special reagents

Sephadex G-50 superfine was from Pharmacia Biotechnology

Abbreviations used: MT, metallothionein; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; e.s.m.s., electrospray mass spectrometry.

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International AB, Uppsala, Sweden. Nucleosil 100 (10 μm particle size) and Nucleosil 300 (5 μm particle size) were from Macherey-Nagel, and Dynosphere PD-102-RE (10 μm particle size) was from Dynosphere Particles A/S.

Pork insulin was from Novo Nordisk Gentofte A/S. *Staphylococcus aureus* V8 proteinase (EC 3.4.21.19) was from Miles Laboratories. Recombinant bovine acyl-CoA-binding protein was produced as described by Mandrup et al. (1991). Dithiothreitol (DTT), phenylmethanesulphonyl fluoride (PMSF) and Trizma base were from Sigma; trifluoroacetic acid of gas chromatography grade and acetonitrile of h.p.l.c. grade were from Rathburn Chemicals.

All other chemicals were of analytical grade and supplied by Fluka Chemika, Riedel deHäen or Merck.

Animals

Shore crabs, *C. maenas* (L.), were caught in Egensedybet, Odense Fjord, Denmark, and kept in large tanks supplied with running seawater with a salinity of 16–23 p.p.t. at the Marine Laboratory, Bøgebjerg, Denmark. Male crabs (carapace width 60–80 mm) were selected and held in well-aerated seawater in polystyrene aquaria (four to five animals per 10-litre aquarium) at constant temperature (15 °C). Seawater was changed at least twice per week. Except for occasional scavenging on dead crabs, experimental animals were starved during the exposure period.

Exposure to Cd²⁺

After 1 day of acclimation to the experimental conditions, the crabs were exposed to Cd²⁺ for 25–35 days. Cd²⁺ (0.5 or 2 p.p.m.) was added to ambient seawater as CdCl₂. Animals that appeared sluggish or exhibited other behavioural abnormalities at the end of the exposure period were discarded. The remaining crabs were killed by mechanical destruction of the ventral nerve ganglion (Baker, 1955). Midgut glands were immediately dissected out and stored in liquid nitrogen. Tissues were held at –80 °C until analysis.

Purification of MT

A sample of frozen midgut gland tissue was placed in a mortar filled with liquid nitrogen and crushed. The resulting powder was transferred to a centrifuge tube. Twice the tissue weight of ice-cold distilled water containing 1 mM DTT and 0.1 mM PMSF was added. After partial thawing the suspension was sonicated for approx. 20 s and additional PMSF was added to achieve a final concentration of 0.2 mM. The homogenate was centrifuged for 90 min at 116000 g at 4 °C. The supernatant was then filtered through glass wool under argon to remove lipids. The filtrate was placed in an iced-water bath and acetone at –20 °C was slowly added with vigorous stirring. Additions were made so that acetone successively comprised 40, 60 and 80 % of the solution. Between each addition the mixture was centrifuged for 15 min at 27000 g at 4 °C. The 60–80 % pellet produced was redissolved in 10 ml of 30 mM Trizma base/HCl, pH 9, containing 1 mM DTT for gel filtration. A Sephadex G-50 column (89 cm \times 2.6 cm) was equilibrated, and samples were eluted with the above buffer at a flow rate of 29.1 ml/h. The column was calibrated with Blue Dextran, insulin (molecular mass 5800 Da) and acetone. The eluate was collected in 20 min fractions while the u.v. absorbance at 254 nm was monitored. The main metal-binding fractions obtained from gel filtration were then pooled and concentrated on an Amicon 202 ultrafiltration system (molecular mass cut-off 5000 Da). The concentrated G-50 fraction was loaded on a Nucleosil 100 (10 μm particle size) h.p.l.c. column (110 mm \times

6 mm), equilibrated with buffer A (50 mM ammonium acetate, pH 6.0). Proteins were eluted with a gradient of buffer B [50 mM ammonium acetate, pH 6.0, in 60 % (v/v) acetonitrile] in buffer A. The gradient was 0–15 % buffer B in 30 min, followed by 15–90 % buffer B in 40 min. The flow rate was 2 ml/min and u.v. absorbance was monitored at 227 nm.

Metal analyses

Cd²⁺, Cu²⁺ and Zn²⁺ concentrations were determined by atomic absorption spectrometry on a Perkin-Elmer 2380 atomic absorption spectrophotometer.

Metal removal and protein modification

Metal was removed by the procedure of Hunziker (1991a). Cysteine was S-methylated as described by Hunziker (1991b).

Digestion with *S. aureus* proteinase

MT (0.5 mg) was dissolved in 0.5 ml of 100 mM ammonium acetate buffer, pH 4.0. *S. aureus* proteinase (50 μg) was added and the digestion was allowed to proceed at 37 °C for 18 h until terminated by injection on h.p.l.c.

Purification of peptides

Peptides were separated on a Nucleosil 300 (5 μm particle size) h.p.l.c. column (120 mm \times 8 mm), equilibrated with solvent A [0.1 % (v/v) trifluoroacetic acid]. Peptides were eluted with a gradient of solvent B [0.1 % (v/v) trifluoroacetic acid in 60 % (v/v) acetonitrile] in solvent A. The gradient was 0–24 % solvent B in 10 min, followed by 24–34 % solvent B for a further 40 min, and 34–60 % solvent B for a further 10 min. The last gradient step was performed in order to wash the column.

Determination of amino acid sequence

Intact protein and peptides were sequenced on a Knauer 810 pulsed-liquid sequencer with chemicals and methods as recommended by the manufacturer: 0.5–2 nmol of protein or peptide was used for each sequence run, and the amino acid phenylthiohydantoin derivatives were identified on an ABI 140B (Applied Biosystems) on-line h.p.l.c. system using a 250 mm \times 2 mm Lichrosphere 100 (5 μm particle size) column and a gradient of acetonitrile in 50 mM sodium acetate buffer, pH 3.8, as described by the manufacturer.

M.s.

The molecular mass of each peptide was determined on a Bio-Ion BIN 20 K plasma-desorption time-of-flight mass spectrometer. The samples were dissolved in 0.1 % trifluoroacetic acid, and 10–100 pmol was applied in 2–3 μl to an aluminized Mylar foil coated with nitrocellulose and spin-dried (Nielsen et al., 1988). After insertion of the sample into the mass spectrometer, it was bombarded with fission fragments from a 10 μCi ²⁵²Cf source. The spectra of peptides were recorded for 5×10^6 primary ions.

The molecular masses of intact MTs were determined by electrospray mass spectrometry (e.s.m.s.). E.s. mass spectra were recorded using a Vestec model 201 single-quadrupole mass spectrometer (Vestec Corporation, Houston, TX, U.S.A.) equipped with an e.s. interface as described (Allan and Vestal, 1992). Protein samples were dissolved in 1 % acetic acid/50 % methanol to a concentration of 5–10 pmol/ μl , and 5 μl were infused by a syringe pump (Orion Research, Boston, MA, U.S.A.) at a flow rate of 0.3 $\mu\text{l}/\text{min}$. Full-scan spectra (m/z 400–1500) were acquired using a Teknivent Vector2 data system

(St. Louis, MO, U.S.A.), and several scans were averaged to obtain the best signal-to-noise ratio. The instrument was independently calibrated using a mixture of arginine and recombinant bovine acyl-CoA-binding protein (Mandrup et al., 1991). Each molecular species produced a series of multiply charged protonated molecular ions from which the molecular mass was determined using the Vector2 electrospray data system. Results are quoted as a mean molecular mass derived from several multiply charged ion peaks. Molecular masses are given as average values based on the relative atomic masses of the elements.

RESULTS AND DISCUSSION

The three-step acetone-precipitation protocol employed in the present work was modified from a method used for the purification of vertebrate metal-binding proteins (Bell and Lopez, 1985; Waalkes and Perantoni, 1986; Waalkes et al., 1988; Baer and Thomas, 1991). Measurements of Zn^{2+} , Cu^{2+} and Cd^{2+} on the Sephadex G-50 gel-filtration fractions obtained from the 60–80% acetone precipitate from crabs exposed to 2 p.p.m. of Cd^{2+} revealed a main metal-binding peak eluted with an apparent molecular mass of 7–10 kDa (Figure 1). The metal peak coincided with a maximum in u.v. absorbance at 254 nm attributable to absorbance by the Cd^{2+} -thiolate complexes.

From the metal contents of the G-50 gel-filtration peak and an assumed molecular mass for MT of 6000 Da, the MT content was calculated as follows: mol of MT = (mol of Cd/6) + (mol of Cu/10) + (mol of Zn/6) (Engel, 1987). Tissue concentrations were estimated from these values (Table 1). When the same method was applied to midgut gland samples from crabs exposed to 0.5 p.p.m. of Cd^{2+} virtually all the Cd^{2+} , Cu^{2+} and Zn^{2+} was eluted in the 7–10 kDa region on gel filtration (results not shown). A Zn^{2+} and Cu^{2+} peak was also observed in the 7–10 kDa range after gel filtration of the 60–80% acetone precipitate of midgut gland from unexposed crabs, whereas Cd^{2+} could not be detected (results not shown). Exposure of crabs to 0.5 p.p.m. of Cd^{2+} gave an MT peak with equimolar amounts of Cd^{2+} , Cu^{2+} and Zn^{2+} (Table 1). After exposure to 2 p.p.m. of Cd^{2+} , the Cd^{2+} content in the 7–10 kDa peak exceeded Zn^{2+} and Cu^{2+} contents by 5- and 17-fold respectively. The calculated MT content increased 5-fold. Similar results were reported by Olafson et al. (1979) after exposure of *S. serrata* to Cd^{2+} (Table 1).

The fractions underlined in Figure 1 were pooled and concentrated and further purified by reversed-phase h.p.l.c. (Figure 2). The proteins separated into a major (MTIa) and two minor (MT0, MTIb) Cd^{2+} -binding peaks eluted at 24, 20 and 29.5 min respectively, and a major broad peak eluted between 34 and 40 min. Measurements of Cd^{2+} in the h.p.l.c. fractions by atomic absorption spectrometry showed that 80% of the recovered Cd^{2+} was bound in the MTIa peak, 6% was bound in MTIb and 14% in the complex eluted between 34 and 40 min.

The purity of the MTIa and MTIb peaks was tested by rechromatography on a Dynosphere h.p.l.c. column at pH 2.2. The MTIa peak was found to contain only one component, whereas the MTIb peak contained a very small amount of MTIa (results not shown). These results were confirmed by e.s.m.s. where only one and two mass peaks for MTIa and MTIb were found respectively (Figure 3). From the e.s.m.s. peaks, the molecular masses of MTIa and MTIb were estimated to be 6001.1 Da and 6133.0 Da respectively. MT0 was an oxidation artifact, as incubation of the concentrated gel-filtration samples with 10 mM DTT overnight at 5 °C before h.p.l.c. completely removed the peak. Attempts to measure a molecular mass of the complex eluted between 34 and 40 min in Figure 2 by e.s.m.s.

revealed a heterogeneous mixture of low-molecular-mass compounds. Except for trace amounts of MTIa, MT-like proteins could not be detected in this peak and it was therefore not investigated further. The h.p.l.c. profile of MT obtained from animals exposed to 0.5 p.p.m. of only Cd^{2+} did not contain the

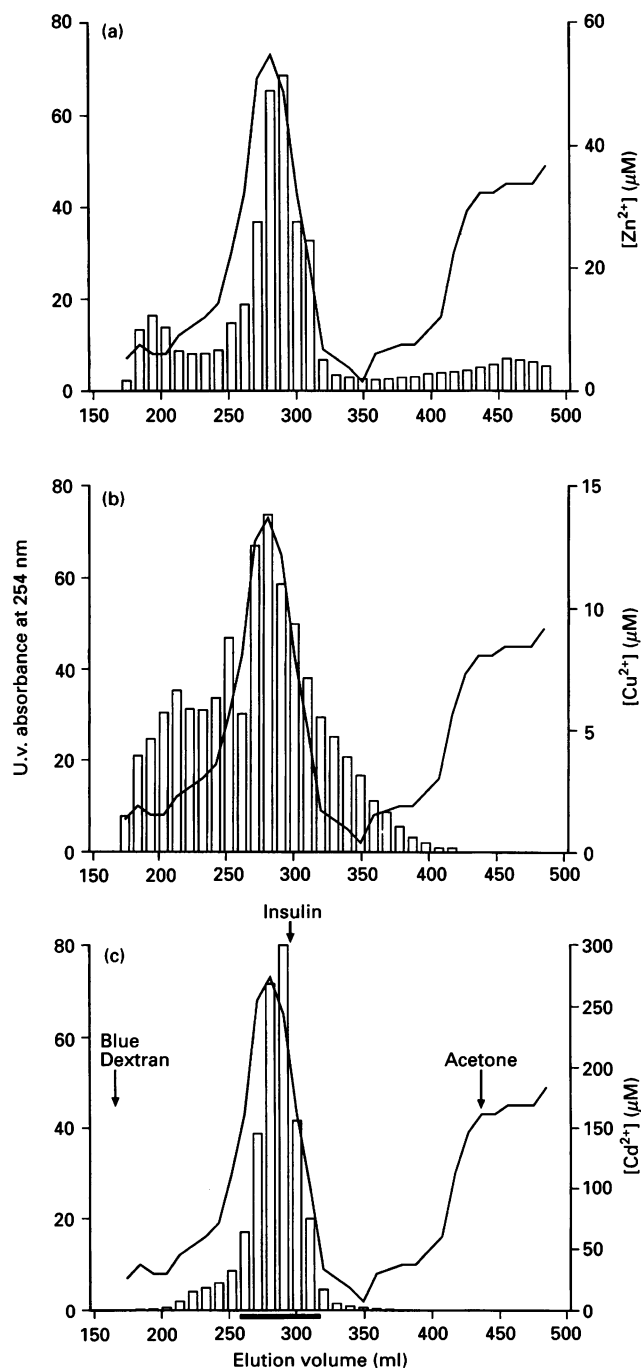


Figure 1 Gel filtration of acetone precipitate obtained from midgut gland tissue from crabs exposed to 2 p.p.m. Cd^{2+}

Elution of (a) Zn^{2+} , (b) Cu^{2+} or (c) Cd^{2+} . The sample was a 60–80% acetone precipitate of 25 g (w/w) of tissue. The column was eluted at a flow rate of 29.1 ml/h. U.v. detection at 254 nm is given in arbitrary units. The underlined fractions (7–10 kDa) were pooled for h.p.l.c. Metals were measured by flame atomic absorption spectrometry. □, Metal concentration; —, u.v. absorbance.

Table 1 Metal contents of the 7–10 kDa gel-filtration fractions (the MT peak) from unexposed crabs, and crabs exposed to 0.5 and 2.0 p.p.m. Cd²⁺ (underlined fractions in Figure 1)

n.d., Not determined.

	Metal content (nmol/g of tissue, w/w)			Ratio Cd ²⁺ /Zn ²⁺ /Cu ⁺	Estimated MT (μg/g)†
	Cd ²⁺	Zn ²⁺	Cu ²⁺		
Control	n.d.	10.1	42.3	0:1:4.2	36
Cd ²⁺ 0.5 p.p.m.	37.0	32.8	45.1	1.1:1:1.4	96
Cd ²⁺ 2.0 p.p.m.	391.0	75.2	23.1	16.9:3.3:1	480
<i>Scylla</i> MTT*	—	—	—	10.5:1.1:1	350
<i>Scylla</i> MTII*	—	—	—	12.7:2.8:1	350

* The data for *Scylla serrata* were recalculated from Olafson et al. (1979).

† MT concentrations in midgut gland from *C. maenas* were estimated by the formula of Engel (1987) (see the text).

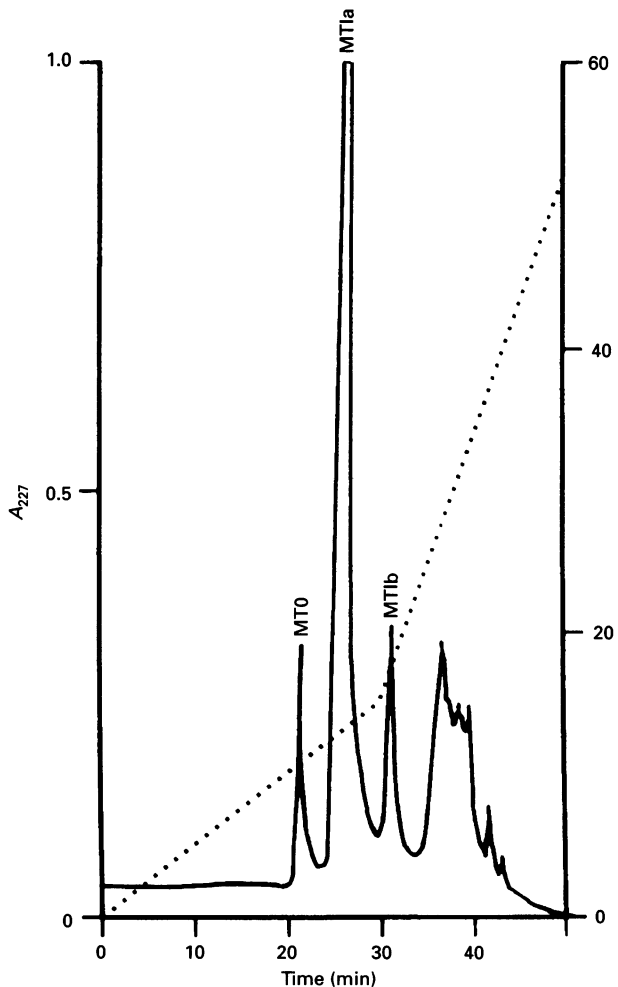


Figure 2 Reversed-phase h.p.l.c. (pH 6.0) of metal-binding fraction from Sephadex G-50 gel filtration

The main Cd²⁺-binding fractions from G-50 gel filtration of acetone-precipitated proteins from midgut gland of crabs exposed to 2 p.p.m. Cd²⁺ were subjected to reversed phase h.p.l.c.; 1 ml was injected [equivalent to 2 g (w/w) of tissue]. U.v. absorbance was detected at 227 nm (—)., % of solvent B. Peaks were eluted at 20 min (MTO), 24 min (MTIa), 29.5 min (MTIb) and 34–40 min (a complex).

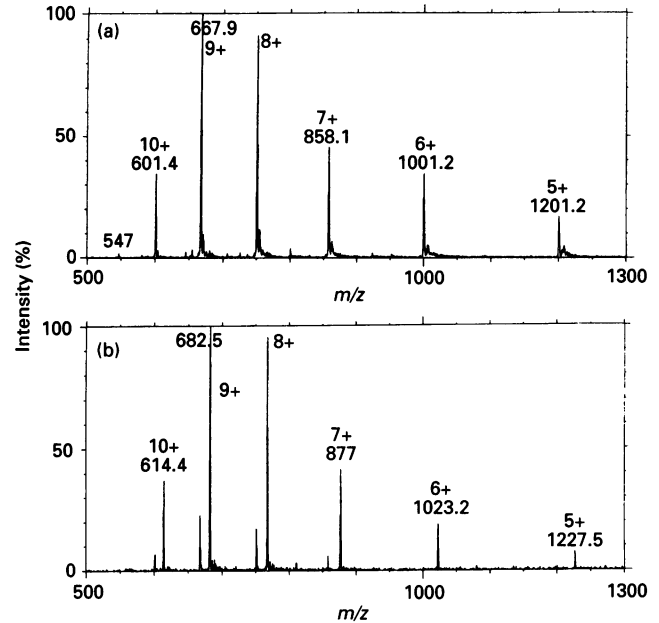


Figure 3 Electro spray ionization mass spectrum of h.p.l.c.-purified MTIa and MTIb from Figure 2

Ionization was performed with an electro spray solution that contained water/methanol/acetic acid (2:2:1, by vol.), pH 2.5. The *m/z* values together with the charge states (10+ to 5+) are shown. The molecular masses of MTIa (a) and MTIb (b) are 6001.1 Da and 6133.0 Da respectively. In the spectrum of MTIb trace amounts of MTIa can be seen.

MTIb peak, but was otherwise identical with the profile shown in Figure 2 (results not shown).

Neither MTIa nor MTIb could be detected in the h.p.l.c. profile when the Cu²⁺- and Zn²⁺-binding 7–10 kDa gel-filtration fraction from unexposed crabs was purified by reversed-phase h.p.l.c. Only the complex eluted between 34 and 40 min was present (results not shown).

Both MTIa and MTIb were susceptible to oxidation. Storage of the purified MT for 2–4 days at 0 °C even in the presence of 1 mM DTT resulted in multiple peaks on rechromatography using h.p.l.c. However, the oxidation could be reversed by incubation in 100 mM DTT, 60 °C for 2–4 h. The high susceptibility of invertebrate MTs to oxidation has previously been reported by Overnell (1986) and Wong and Rainbow (1986), and complicates their purification by reversed-phase h.p.l.c. Schlenk and Brouwer (1991) tried to circumvent the problem by modifying crab MT before the h.p.l.c. step.

The complete sequence of MTIa and MTIb from *C. maenas* is given in Figure 4. The proteins contained 57 and 58 residues respectively and, like other crustacean MTs, both proteins contain an unblocked N-terminus (Lerch et al., 1982; Brouwer et al., 1989). The masses calculated from the obtained sequences were 6001.9 Da for MTIa and 6133.1 Da for MTIb, which agree well with the masses 6001.1 Da and 6133.0 Da determined for MTIa and MTIb respectively by e.s.m.s. (Figure 3). MTIa and MTIb were identical except for an additional N-terminal methionine in MTIb (Figure 4). The sequence of the first 45 N-terminal residues of MTIa was obtained directly by sequencing the intact *S*-methylated proteins. Digestion of *S*-methylated MTIa with *S. aureus* proteinase at pH 4 cleaved the protein into four peptides, cleavage occurring after Glu-10, Glu-13 and Glu-44. No cleavage was observed after Glu-31, probably because of the neighbouring basic lysine residue. The peptides were isolated

(Met) - Pro-Asp-Pro-Cys-Cys-Ile-Asp-Lys-Cys-Glu-Cys-Lys
 Sa1
 Glu-Gly-Gly-Cys-Lys-Ala-Gly-Cys-Lys-Cys-Thr-Ser
 Sa3
 Cys-Arg-Cys-Thr-Pro-Cys-Glu-Lys-Cys-Ser-Ser-Gly
 Gly-Cys-Lys-Cys-Thr-Thr-Lys-Glu-Asp-Cys-Cys-Lys
 Sa4
 Lys-Thr-Cys-Thr-Lys-Pro-Cys-Ser-Cys-Cys-Pro

Figure 4 Primary structure of MTIa and MTIb from *C. maenas* and the peptides used to construct the amino acid sequence

S. aureus proteinase cleavage sites (pH 4.0) are marked with arrows, and proteinase-generated peptides are shown with the prefix Sa. The peptides are numbered from the N-terminus. MTIb contains the N-terminal residue of methionine shown in parentheses.

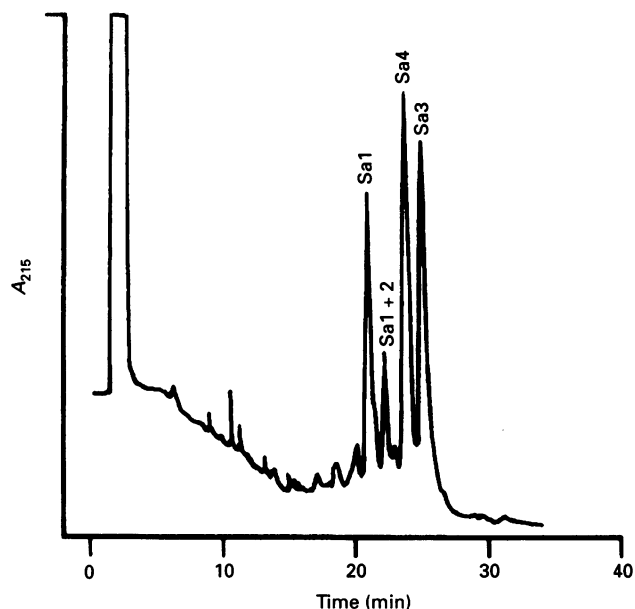


Figure 5 *S. aureus* proteinase-produced peptide map of h.p.i.c.-purified MTIa

The peptide map of *S*-methylated MTIa from *C. maenas* exposed to 2 p.p.m. of Cd²⁺ was separated on a Nucleosil 300 (5 μm particle size) column as described in the Materials and methods section. The gradient was 0–24% solvent B in 10 min, followed by 24–34% solvent B from 10–50 min. The numbering of the peptides refers to Figure 4.

Table 2 Expected and observed masses of modified peptides from *S. aureus* proteinase digestion of MTIa

Observed masses were determined by plasma-desorption m.s. as described in the Materials and methods section. The numbering of peptides refers to Figure 4. n.d., not determined.

Peptide	Expected mass (Da)	Observed mass (Da)
Sa1	1164.3	1165.6
Sa2	392.4	n.d.
Sa1 + 2	1538.7	1539.2
Sa3	3189.6	3189.6
Sa4	1575.8	1577.0

by h.p.i.c., except for the tripeptide Sa2, which could not be recovered (Figure 5). The masses of the isolated peptides were determined by plasma-desorption m.s. and were found to be in

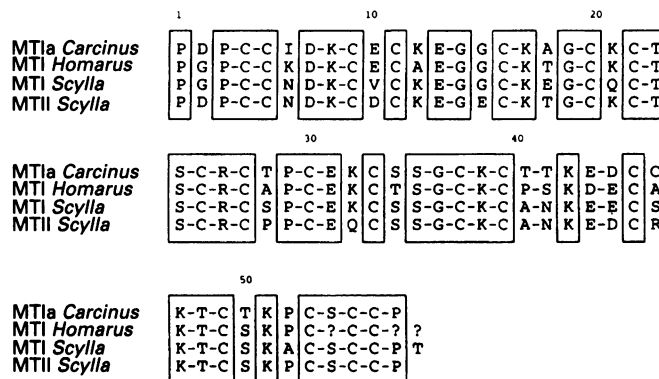


Figure 6 Amino acid sequences of three decapod crustacean MTs

Conserved residues are shown in boxes. *Scylla*, *Scylla serrata* (Lerch et al., 1982); *Homarus*, *Homarus americanus* (Brouwer et al., 1989); *Carcinus*, *Carcinus maenas* (this paper).

good agreement with the expected masses calculated from the sequence (Table 2). Edman degradation of Sa1 and Sa3 was entirely consistent with the sequence obtained from the intact protein. The remainder of the sequence was obtained by Edman degradation of Sa4. The sequence of MTIb was determined using the same procedure as described above.

Compared with other crustacean MTs, the 18 cysteines of *C. maenas* MT are highly conserved (Figure 6), thus establishing the protein as a class 1 MT (Fowler et al., 1987). *C. maenas* MT contains an additional cysteine in the C-terminal half of the molecule at position 46. The hypervariability of this particular position might indicate that little if any functional significance should be attached to the additional cysteine. However, the cysteine substitution resulted in the formation of an additional Cys-Cys motif which is a characteristic feature of MTs (Kägi and Kojima, 1987). Nearly all basic amino acids (lysines and arginine) are conserved among crustacean MTs (Figure 6), and seven of nine lysines appeared in juxtaposition to a cysteine. ¹H n.m.r. has shown that the ε-amino groups of lysines are markedly stabilized when MT is complexed with metal, suggesting that hydrogen-bond-mediated electrostatic interactions occur between the protonated basic residues and the negatively charged metal-thiolate complex (Vašák et al., 1985).

In general, decapod crustacean MTs appear to be highly conserved. Except for two hypervariable single-residue sites (positions 28 and 46), only a few conservative exchanges occur. It is notable that the degree of identity between *C. maenas* MTIa and *S. serrata* MTII is higher (83%) than the identity between the two isoforms from *S. serrata* (81%).

When *C. maenas* was exposed to high Cd²⁺ concentrations (2 p.p.m.), about 10% of the MT was the MTIb variant which had an additional N-terminal residue of methionine. During exposure to the lower Cd²⁺ concentration, the MTIb variant was absent, indicating that high concentrations of Cd²⁺ either inhibited the processing enzymes and/or that the processing enzymes could not keep pace with the increased MT synthesis. Most vertebrate MTs, the primary structure of which has been determined, possess an N-terminal methionine which can be either blocked or unblocked in contrast with decapod crustacean MTs in which the N-terminal methionine is processed (Kägi and Kojima, 1987).

Two main MT isomers which are charge-separable and coded for by different genes can be found in most species (Kägi and Kojima, 1987). Among the decapod crustaceans, amino acid

analysis indicates the existence of multiple isomers in the Cd²⁺- and Cu²⁺-treated crab (*Callinectes sapidus*) (Brouwer et al., 1992) and in Cu²⁺-treated lobster (*H. americanus*) (Brouwer et al., 1989), but only in the case of the crab *S. serrata* (Lerch et al., 1982) has the presence of more than one isomer been confirmed by sequence analysis. The Cd²⁺-exposed shore crab *C. maenas* investigated in the present study appears to have only one isomer. A variant of this isomer, with an N-terminal methionine, is found when Cd²⁺ exceeds a particular threshold concentration. Overnell (1986) also found only one isomer in the Cd²⁺-exposed crab *Cancer pagurus*, although this was not confirmed by sequence analysis. Interestingly, amino acid analysis indicates that the *Cancer pagurus* MT resembles *C. maenas* MT in that it contains 19 cysteine residues in contrast with the 18 cysteine residues reported for MTs in other crustaceans (Lerch et al., 1982; Brouwer et al., 1989).

The existence of an increasing number of species which apparently have only one MT isomer (McCormick et al., 1988; Lin et al., 1990; George et al., 1992; Chan et al., 1989) casts doubt on the frequently postulated physiological importance of having multiple MT forms (Nath et al., 1988; Brouwer et al., 1992). Differences in the physiological roles of MT isomers have been suggested on the basis of tissue- and cell-type-specific expression of isomers (Nomiya and Nomiya, 1982; Richards et al., 1984; Sadhu and Gedamu, 1988; Jahroudi et al., 1990), different rates of isomer induction by different metals (Brouwer et al., 1992), different rates of induction with metals, endotoxins and hormones (Richards et al., 1984; Abe et al., 1987; Foster and Gedamu, 1991), different rates of degradation of isoforms (Mehra and Bremner, 1985; Klaassen and Lehman-McKeeman, 1989) and different ratios of MT isoforms during growth and development (Suzuki et al., 1983; Cain and Griffiths, 1984; Klaassen and Lehman-McKeeman, 1989). Related species expressing different numbers of MT isomers in response to metal exposure could be useful model systems for studying the physiological importance of MT polymorphism. For example, the closely related crabs *C. maenas* and *Callinectes sapidus* apparently express one and two MT isomers respectively in response to exposure to Cd²⁺ (Brouwer et al., 1992; this paper).

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