Primary structures of decapod crustacean metallothioneins with special emphasis on freshwater and semi-terrestrial species

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Cadmium injections induced only a single form of metallothionein (MT) in the midgut gland of *Potamon potamios*, whereas the same treatment induced two isoforms in *Astacus astacus*. The only difference between the two latter isoforms was that one had an extra N-terminal methionine residue. MT from *P. potamios* showed structural differences from other decapod crustacean MTs. It contained a Gly-Thr motif at positions 8 and 8a, which had previously been found only in certain vertebrate and molluscan MTs. Furthermore *P. potamios* MT contained two to three times as many glutamic acid residues as normally found in decapod crustacean MT. The primary structure of MT from the freshwater crayfish *A. astacus* showed a high degree of sequence identity with MT from other decapod crustaceans, especially the marine astacidean *Homarus americanus*, although two valine residues were unexpectedly found at positions 8 and 21, where lysine residues are normally found.

INTRODUCTION

Metallothionein (MT) is a small inducible sulphur-rich metal-binding protein found intracellularly in most organisms [1]. In many species the protein can be induced from very low basal levels to levels exceeding several mg/g of tissue by a wide variety of factors, including metals, alkylating agents, irradiation, infection and various physical stresses [1–3].

However, after more than 30 years of extensive research, the precise physiological role (or roles) of MT is still not clear. Recent experiments with transgenic mice and cell lines with disrupted genes for MT indicate that the protein might be involved in the detoxification of cadmium [4], in embryonic development [5], in protection against oxidative stress [6] and in the acute-phase response [7,8]. Furthermore a role in homeostasis of the essential metals zinc and copper, and in metal ion donation/activation of apo-metalloenzymes has been suggested [9,10].

Mammalian MTs consist of 61 or 62 amino acid residues, of which 20 are cysteine residues. They are frequently found in several isoforms coded by non-allelic genes [1]. Among invertebrates, only mollusc MTs and decapod crustacean MTs show sequence similarities to vertebrate MTs (class I MT). In the early 1980s the complete primary structure of MT I and II from the crab *Scylla serrata* was published [11]. The two isoforms contained 58 and 57 amino acid residues respectively and showed a sequence identity of 83 %. Furthermore partial sequences of the MT from the lobster *Homarus americanus* [12], the complete sequence of one MT isoform from the shore crab *Carcinus maenas* [13] and two isoforms from the blue crab *Callinectes sapidus* [14] have been reported. The four crustaceans mentioned above all inhabit the marine environment and show a high degree of sequence homology in their MT isoforms.

In the present study, MTs from two decapod crustaceans not occurring in the marine environment have been characterized. The N-terminal sequence of MT from the crab *Potamon potamios* is compared with N-terminal sequences from both vertebrate and invertebrate species, and similarities are discussed. In addition, both MT isoforms from *P. potamios* and *Astacus astacus* are compared with the sequences already known for MT from marine decapod crustaceans to gain a holistic view of MT structure in marine, freshwater and semi-terrestrial decapod crustaceans.

EXPERIMENTAL

Enzymes and special reagents

Superose 12 was from Pharmacia Biotechnology International AB. Nucleosil 100 (5 and 10 μm particle size) and Nucleosil 300 (5 μm particle size) were from Macherey-Nagel. Cd-MT from *Car. maenas* was produced as described by Pedersen et al. [13]. *Staphylococcus aureus* V8 proteinase (EC 3.4.21.19) was from Miles Laboratories. Carboxypeptidases Y and P (EC 3.4.16.1) were from Boehringer Mannheim. Dithiothreitol (DTT) and PMSF were from Sigma Chemical Corporation; acetonitrile of HPLC grade was from Rathburn Chemicals. All other chemicals were of analytical grade, and supplied by Fluka Chemika, Riedel deHäen or Merck.

Animals

Freshwater crayfish, *A. astacus*, were purchased from a local breeder and kept in artificial fresh water in well-aerated polystyrene aquaria (four or five animals per 10 litre aquarium) at a constant temperature of 15 °C. Semi-terrestrial crabs, *P. potamios*, were caught in Lake Cornas on the island of Crete (Greece) and maintained in tanks containing a few centimetres of tap water at a constant temperature of 15 °C. Only large males in intermoult condition were used.

Induction of MT in *A. astacus* and *P. potamios*

The animals were injected with increasing amounts of $CdCl_2$ in physiological saline. The dosing regime was (in mg Cd/kg live

Abbreviations used: DTT, dithiothreitol; MT, metallothionein.

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weight): day 1, 0.25; day 6, 0.5 ; day 10; 1.0 ; day 15, 2.0; day 20, 4.0. The animals were killed 4 days after the last injection. Midgut glands were immediately dissected out and stored in liquid nitrogen. Tissues were held at −80 °C until analysis of MT.

Metal analysis

Cadmium was measured by atomic absorption spectrophotometry on a Perkin Elmer 2380 atomic absorption spectrophotometer.

Purification of MT

Frozen midgut gland tissue was crushed in a mortar filled with liquid nitrogen. The ground tissue was transferred to a 10 ml beaker and 3 vol. (v/w) of ice-cold distilled water containing 1 mM DTT and 0.1 mM PMSF was added. The suspension was sonicated three times for 15 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 resulting supernatant was placed in an ice–water bath and acetone (−20 °C) was slowly added while stirring and purging with argon gas. Acetone was added so that it successively comprised 50 % and 80 % (v/v) of the solution. Between each addition the mixture was centrifuged for 15 min at 27000 ***g*** at 4 °C. The 50–80 % pellet was redissolved in 1 ml of 100 mM NH_4HCO_3/1 mM DTT (pH 8).

The dissolved 50–80 % pellet was loaded on a Superose 12 HR 10/30 gel-filtration column equilibrated with 100 mM ammonium hydrogen carbonate/1 mM DTT (pH 8.0), flow rate 0.6 ml/min, and eluted with the same buffer. A_{254} and A_{280} were monitored continuously. The column was calibrated with Blue Dextran, Cd-MT from the shore crab *Car. maenas*, and acetone. The peak corresponding to crab MT, which was also the main Cd peak, was collected and loaded on a Nucleosil 100 (10 mm particle size) HPLC column (110 mm × 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 used for *A. astacus* MT was 0–30 % buffer B in 60 min, followed by 30–90 % buffer B in 10 min. The gradient used for *P. potamios* MT was 0–15 % buffer B in 30 min, followed by 15–90 % buffer B in 40 min. The flow rate was 1 ml/min and A_{254} was monitored.

Metal removal and protein modification

Metal was removed from the various MT isoforms by the procedure described by Hunziker [15] and cysteine residues were S-methylated by the method of Hunziker [16].

Digestion of *P. potamios* MT

S-methylated apothionein (0.5 mg) was dissolved in 0.5 ml of 100 mM ammonium acetate buffer, pH 4.0. *Staph. aureus* proteinase (25 μg) was added, and the digestion continued at 37 °C for 18 h until terminated by injection into the HPLC column.

Peptides were separated on a Nucleosil 300 (5 μm particle size) HPLC column (120 mm × 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 0–15 % solvent B in 10 min, followed by 15–30 % solvent B in 45 min. The flow rate was 1 ml/min and A_{220} was monitored.

Digestion of *A. astacus* MT

S-methylated apothionein (0.5 mg) was digested with *Staph. aureus* proteinase (25 μg) as described above for *P. potamios* MT. Non-derivatized apothionein (0.5 mg) was dissolved in 0.5 ml of 100 mM NH_4HCO_3 buffer, pH 8.0. *Staph. aureus* proteinase (25 μg) was added, and the digestion continued at 37 °C for 4 h until terminated by injection into the HPLC column. Peptides were separated on a Nucleosil 100 (5 μm particle size) HPLC column (250 mm × 4 mm) equilibrated with solvent A. Peptides were eluted with a gradient of solvent B in solvent A. The gradient was 0–60 % solvent B in 60 min, followed by 60–100 % solvent B in 10 min. The flow rate was 1 ml/min and A_{220} was monitored.

Non-derivatized apothionein (25 μg) was dissolved in 20 μl of 100 mM ammonium acetate buffer, pH 6.0, and carboxypeptidases Y and P were added to give a final enzyme-to-substrate ratio of 1 : 50 (w/w). Aliquots were withdrawn after 0.5, 1, 1.5, 2, 6, 10 and 20 min. The fractions were freeze-dried, redissolved and analysed by laser desorption MS.

Amino acid sequencing

Amino acid sequencing of intact proteins and *Staph. aureus* proteinase-produced peptides were performed as described by Pedersen et al. [13] except that a Knauer 910 pulsed-liquid sequencer was used instead of a Knauer 810.

Laser desorption MS

The matrix-assisted laser desorption mass spectra were acquired on a Bruker Reflex (Bruker-Franzen Analytik) mass spectrometer modified for laser desorption. The matrix used was acyano-4-hydroxycinnamic acid (20 μg/ml in acetone). The sample was prepared by depositing 1 μl of matrix, 0.6 μl of 2 % trifluoroacetic acid, 0.4 μl of sample and finally 0.2 μl of matrix on the aluminium target, followed by drying at ambient temperature. The laser desorption spectra were acquired as single-shot spectra; 20–50 spectra were then averaged.

RESULTS AND DISCUSSION

Superose-12 gel filtration of the dissolved pellet from the acetone precipitation of digestive glands from both *A. astacus* and *P. potamios* gave cadmium-binding peaks eluting in the same position as reverse-phase HPLC-purified *Car. maenas* MT (results not shown). The cadmium-containing MT fractions were pooled and separated by reverse-phase HPLC. Whereas the MT-fraction from *P. potamios* contained only a single MT isoform (Figure 1b) the fraction from *A. astacus* was resolved into two isoforms (Figure 1a). MS showed that the difference between MT 1a and MT 1b in Figure 1(a) was 131 Da (Table 1). Two MT variants were also found in *Car. maenas* treated with cadmium. The difference between the variants was a single methionine residue (mass 131 Da) at the N-terminus [13]. The same difference was seen between the two cadmium-induced MT isoforms isolated from *Cal. sapidus* [14]. Why this pro-MT variant occurs is unknown. It might signify that cadmium inhibits the processing enzymes or that the rate of MT synthesis is higher than the processing rate [13]. In relation to this, it is interesting that the methionine variant was not found in *P. potamios*, even though they were injected with the same amount of cadmium per kg of body weight as *A. astacus*.

The first 36 amino acids from the N-terminus of *P. potamios* MT 1 were obtained by Edman degradation of the intact protein.

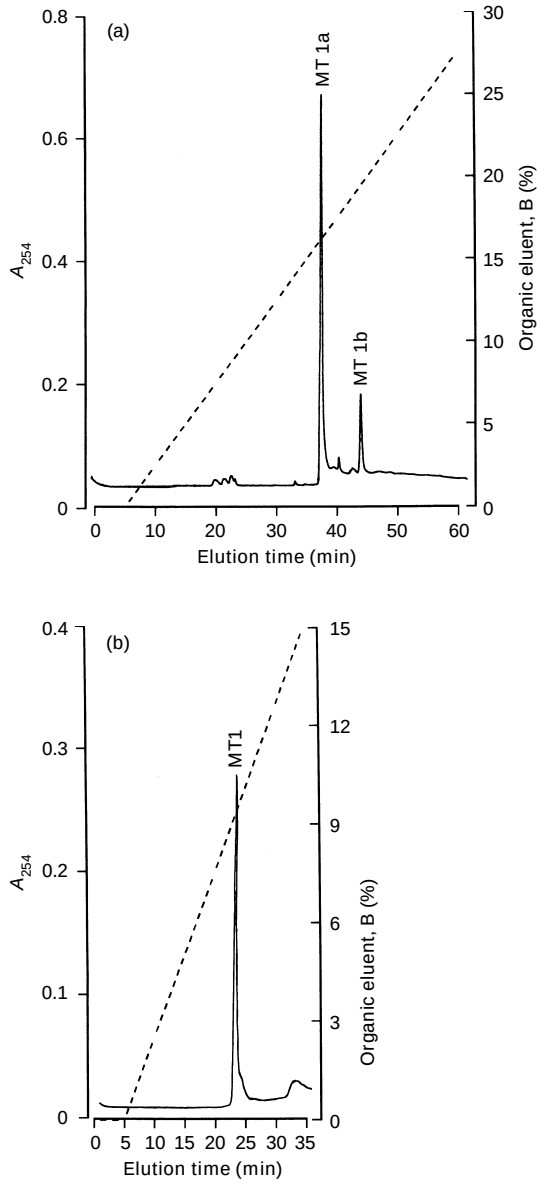

Figure 1 Reverse-phase HPLC elution profiles of MT isoforms from *A. astacus* and *P. potamios*

The peaks, corresponding to crab MT, from Superose-12 gel filtration of acetone-precipitated midgut glands were subjected to reverse-phase HPLC on a Nucleosil 100-10 column. (**a**) *A. astacus* MT contains two variants eluting at 37.8 min (MT 1a) and 44.5 min (MT 1b); (**b**) *P. potamios* MT 1 was eluted at 22 min. Solid line, A_{254}; broken line, percentage of solvent B.

Table 1 Expected and observed molecular masses of MTs from *P. potamios* and *A. astacus*

The expected molecular masses are based on the determined sequences shown in Figure 2; the observed molecular masses were determined by laser desorption MS.

	Expected molecular mass (Da)	Observed molecular mass (Da)
P. potamios MT 1	6156.90	6156.80
A. astacus MT 1a	5779.60	5779.50
A. astacus MT 1b	5910.60	5910.80

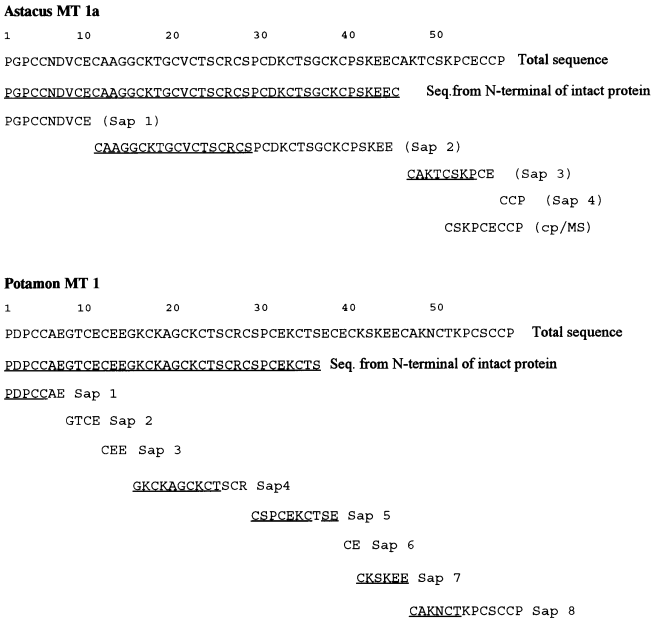

Figure 2 Primary structures of MT 1a from *A. astacus* (Astacus) and MT 1 from *P. potamios* (Potamon)

Proteinase-generated peptides used for sequence determination are shown with the prefix Sap. The peptides are numbered from the N-terminus. Underlined sequences were identified by Edman degradation. The sequence indicated by (cp/MS) was identified by carboxypeptidase digestion and MS. In *P. potamios* the Thr residue after Gly-8 is called 8a.

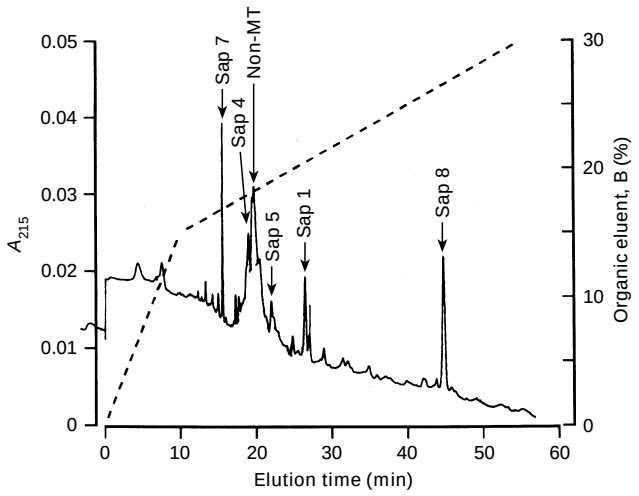

Figure 3 *Staph. aureus* proteinase peptide map of S-methylated MT from *P. potamios*

The peptide map of S-methylated MT 1 from cadmium-exposed *P. potamios* was separated on a Nucleosil 300-5 column as described in the Experimental section. The gradient was 0–15 % solvent B in 10 min followed by 15–30 % solvent B from 10 to 55 min. The numbering of the peptides refers to Figure 2.

The sequences of the peptides used to construct the remaining primary structure of *P. potamios* MT are shown in Figure 2. Digestion of S-methylated MT from *P. potamios* with *Staph. aureus* proteinase at pH 4 resulted in five peptides after separation of the digest by reverse-phase HPLC (Figure 3). The three

Table 2 Expected and observed molecular masses of non-derivatized peptides from *Staph. aureus* proteinase digestion of MT 1a from *A. astacus*

The expected molecular masses are based on the determined sequences shown in Figure 2; the observed molecular masses were determined by laser desorption MS. The differences between the expected and observed molecular masses are due to the formation of disulphide bonds between cysteine residues during protease digestion at neutral pH. Abbreviation: n.d., not determined.

Peptide	Expected molecular mass (Da)	Observed molecular mass (Da)	Number of cysteine residues
Sap 1	1036.10	1034.30	3
Sap 2	3406.90	3398.10	10
Sap 3	1069.30	1067.20	3
Sap 4	321.40	n.d.	2

Human	MT-1a	MDPNCSCAT.GGSCTC	(Reviewed in [1])
	MT-1k	MDPNCSCAAAGVSCTC	(Reviewed in [1])
Plaice	MT	MDP.CECSK.TGTCNC	(Reviewed in [1])
Oyster	MT	MSDPCNCIE.TGTCAC	(Reviewed in [1])
Potamon	MT 1	.PDPC.CAE..**GT**CEC	(This study)
Astacus	MT 1	.PGPC.CND...VCEC	(This study)
Carcinus	MT 1	.PDPC.CID...KCEC	[13]
Scylla	MT I	.PGPC.CND...KCVC	[11]
Scylla	MT II	.PDPC.CND...KCDC	[11]
Callinec.	MT I	.PGPC.CND...KCVC	[14]
Homarus	MT I	.PGPC.CKD...KCEC	[14]

Figure 4 N-terminal sequences from mammalian, fish, mollusc and crustacean MT isoforms

The N-terminal sequence of MTs from human, plaice, oyster, semi-terrestrial crab *P. potamios*, freshwater crayfish *A. astacus*, three marine crabs *Car. maenas*, *Scylla serrata*, *Cal. sapidus* and marine lobster *H. americanus*. The GT motif in the sequence of the crab *P. potamios* is in bold type.

smallest (Sap 2, Sap 3 and Sap 6) could not be recovered. Sap 5 was not a true Sap peptide because it was produced by cleavage after Arg-26, probably owing to contamination with either trypsin or Arg-C proteinase. The recovery of peptides after digestion was very low. This might reflect the fact that not only cysteine residues but also lysine residues were methylated. The excess derivatization could give rise to problems with cleavage owing to steric hindrance. Furthermore the solubility of the resulting lipophilic peptides might be lowered. Only the first six residues of the C-terminal peptide Sap 8 could be obtained by Edman degradation whereas the remainder were constructed by comparison and alignment with already known and conserved sequences from other decapod crustaceans. The sequence of Sap 8 was verified by comparing the calculated and measured masses of this peptide (1427.6 and 1428.5 ± 1.4 Da respectively). The remainder of the sequence was determined by Edman degradation of the various peptides. It was not possible to determine Cys-37 and Glu-38 by Edman degradation, so the identity of these two residues is based on the following evidence. All cysteines are conserved in decapod crustaceans (see Figure 5) and cleavage was observed after position 38 with *Staph. aureus* proteinase. In addition, amino acid analysis confirmed nine Glu residues in the intact protein (results not shown) and with Cys and Glu at positions 37 and 38 the molecular mass of the intact protein (calculated from the sequence in Figure 2) would be 6156.9 Da compared with the measured molecular mass of 6156.8 Da (Table 1).

Intact S-methylated *A. astacus* MT was used to obtain the first 45 residues from the N-terminus (Figure 2). The sequences of the peptides used to construct the remaining primary structure of *A. astacus* MT are shown in Figure 2. To avoid the low recovery of lipophilic peptides, which occurred with *P. potamios* MT, the identity of non-cysteine residues in *A. astacus* MT were established by Edman degradation on peptides derived from SAP digestion of underivatized *A. astacus* MT. In Table 2 the expected peptide masses are compared with the observed masses. The differences between the two sets of masses were caused by the formation of disulphide bonds between cysteine residues owing to the digestion of metal-stripped underivatized MT at neutral pH. Finally, a combination of carboxypeptidases Y and P was used to obtain the C-terminal sequence. The molecular mass of the intact protein was 5779.5 Da and the calculated molecular mass from the amino acid sequence was 5779.6 Da (Table 1).

In Figure 4 the N-terminal sequences of all the currently known decapod crustacean MTs are compared with the N-terminal sequence of vertebrate (human and fish) and invertebrate (mollusc) MTs. *P. potamios* MT differs from all other crustacean MTs in having a Gly-Thr motif at positions 8 and 8a instead of a highly conserved Lys at position 8. The same motif is found in MT from another invertebrate (oyster) as well as in MT from a lower vertebrate *Pleuronectes platessa* (flatfish). Moreover the Gly-Thr or the homologous Gly-Ser motif is commonly found in both lower and higher vertebrates [1]. The discovery of the Gly-Thr motif in both invertebrates and vertebrates suggests that it is an ancient motif that for unknown reasons is missing in most decapod crustaceans. Its reappearance in the modern crustacean family Potamidae [17] might be explained by a mutational activation of the promotor in a silent pseudo-gene containing the Gly-Thr motif. If this is so, this pseudo-gene would then be expected to be present, but silent, in other crustaceans. Further evidence for this hypothesis awaits the characterization of the crustacean MT genome.

In Figure 5 the amino acid sequences of the known decapod crustacean MTs are compared. It is interesting that *P. potamios* MT contains nine glutamic acid residues, two to three times as many as found in other crustacean MTs. This high content of glutamate results in a much higher hydrophilicity of *P. potamios* MT and changed elution behaviour on reverse-phase HPLC. *P. potamios* MT was eluted at 5.1% acetonitrile, compared with the elution of *A. astacus* MT1a at 9.8% (Figure 1). The increased number of glutamic acid residues is found in position 6, where it replaces a highly conserved Asp, in position 36, where Glu replaces a highly conserved Gly, and in position 38, where Glu replaces a highly conserved Lys. In most MTs lysine residues seem to be in juxtaposition with cysteine residues, and ^{1}H NMR has shown that the ϵ-amino groups of lysine residues 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 [18]. In *P. potamios* MT a Lys at position 40 could probably substitute for the missing Lys at position 38. The Lys found in position 15 instead of a conserved Gly probably substitutes for that missing at position 8. Finally, in *P. potamios* MT a highly conserved Thr at position 48 is replaced with an Asn. On the basis of the above observations it seems that although the function of *P. potamios* MT is similar to other MTs, the folding of the protein might differ from that in other crustacean MTs.

As regards MT from *A. astacus*, the differences from other crustacean MTs are not as marked as for *P. potamios* MT. Nevertheless at positions 13 and 54 two highly conserved Glu and Ser residues are replaced by an Ala and a Glu respectively.

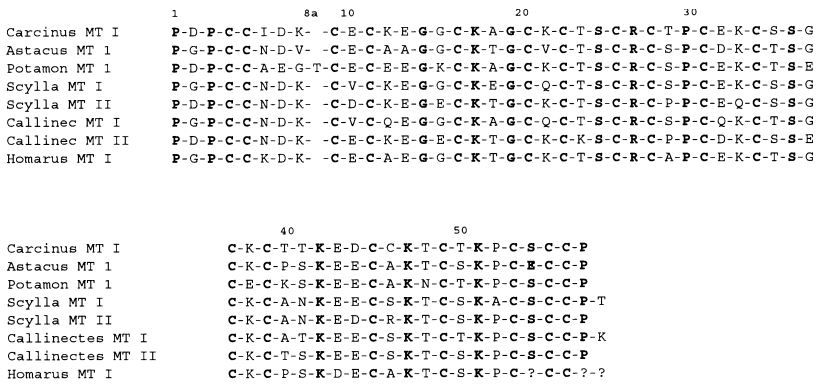

Figure 5 Amino acid sequences of decapod crustacean MTs

Conserved residues are in bold type. Carcinus, *Car. maenas* [1], Astacus, *A. astacus* (this paper); Potamon, *P. potamios* (this paper), Scylla, *Scylla serrata* [11]; Callinectes, *Cal. sapidus* [14]; Homarus, *H. americanus* [12]. The amino acid numbering is according to the consensus; in *P. potamios* the Thr residue after Gly-8 is called 8a.

Furthermore at positions 8 and 21 two Lys residues are replaced by two Val residues (Figure 5). The latter exchanges bring down the total lysine content to six residues, compared with a normal content of eight or nine (Figure 5). This is a surprisingly small number considering the electrostatic requirements for cancelling out negative charges in the metal clusters as mentioned above. The characterization of MT from *A. astacus* was initially performed because no data were available on freshwater crustaceans. Furthermore the MT sequence from another astacidean, the marine *H. americanus* [12], had already been published. We therefore considered that it would be interesting to see whether MT from two species from the same infraorder inhabiting different environments showed any structural differences. In the MT sequence from *H. americanus* two amino acid residues at positions 54 and 57 remain to be identified, but assuming that they are identical to Glu-54 and Pro-57 found in *A. astacus* MT, the sequences from *A. astacus* and *H. americanus* differ only in seven positions, of which two are homologous exchanges of Glu for Asp, yielding 88% identity. For comparison, the sequence identity between two MT isoforms from a single species (mud crab, *Scylla serrata*) is only 81% [11]. Especially the C-terminal ends from positions 32–57 in *A. astacus* and *H. americanus* are identical, with the exception of a homologous exchange of Glu-43 in *A. astacus* for Asp-43 in *H. americanus*.

In the present work we characterized for the first time MTs from semi-terrestrial and freshwater decapod crustaceans. Compared with the already existing primary structures of MTs from marine decapod crustaceans the following features seem to be common. They contain 57 of 58 amino acid residues, of which 18 are conserved cysteines arranged in five Cys-Xaa-Cys-, two Cys-Cys- and three Cys-Xaa-Yaa-Cys- motifs, establishing the proteins as class 1 MTs [19]. Most have eight or nine lysine residues to fulfil the electrostatic requirements; only *A. astacus* MT has fewer (six Lys). The N-terminus is an unblocked Pro-Xaa-Pro-Cys-Cys motif, where Xaa is either Asp or Gly. A highly conserved region from Ser-24 to Cys-27 is always found, making the region suitable as a template for constructing DNA primers. In addition, MT from *P. potamios* showed structural differences that have not been observed so far in any other decapod MT. It could be interesting to see whether other members of the family Potamonidea, or other crustaceans in general, show the same structural similarities as vertebrate MTs.

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