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Isolation and characterisation of metallothionein from the clam *Ruditapes decussatus*

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Abstract

Metallothioneins (MT) were obtained after purification from metal-exposed clams (*Ruditapes decussatus*) using gel-permeation and ion-exchange chromatography. Four cadmium–metallothioneins (CdMTs) were resolved by ion-exchange chromatography and they all had similar molecular weights, high cadmium content and an absorption spectra indicative of the presence of characteristic Cd–S aggregates. The NH_2-terminal sequence suggests the presence of at least two class I clam MT isoforms. For the other two putative clam CdMTs isolated, the results of the amino acid determination were inconclusive. One was slightly contaminated and the other one had a blocked NH_2-terminal. These clam metalothioneins contain glycine, which seems to be a common feature of molluscan MT family and exhibited more similarity to oysters than to mussels. Further investigation on the inducibility of these isoforms will be necessary if clams are to be used as biomarkers of metal exposure.

Keywords: Metallothionein; Clam; *Ruditapes decussatus*; Cd; Toxicity

1. Introduction

Despite their discovery by Margoshes and Vallee more than 40 years ago in 1957, metallothioneins (MTs) are still an interesting matter of research (see Kagi, 1993; Roesijadi, 1993; Langston et al., 1998; Dabrio et al., 2002 for reviews). These proteins are low-molecular mass (6–7 kDa; 57–75 amino acids), cysteine-rich proteins (18–20 cysteines per molecule) lacking aromatic amino acids (Kagi and Shaffer, 1988). They are distinguished by an exceptionally high content of d^{10} metal ions (Zn^{2+}, Cd^{2+} and Cu^{+}) forming characteristic metal–thiolate clusters through the sulphur atoms of all the cysteine groups that compose the protein, with thiolate groups acting both as a terminal and bridging metal ions (Dabrio et al., 2002). MT is a cytosolic protein found in a variety of prokaryotic and eukaryotic organisms including vertebrates, invertebrates, plants and microorganisms where the occurrence of the amino acid sequence Cys-Xaa-Cys is a character-

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istic feature of these proteins (Hammer, 1986; Kagi and Kojima, 1987a,b). MTs occurs in several isoforms which may differ from each other by only a few amino acid positions and by different isoelectric points and hydrophobicity (Kagi, 1993; Dabrio et al., 2002). The existence of different MT isoforms with different roles has also been a matter of scientific interest (Nordberg, 1998).

The function of this protein and of its isoforms is still a matter under discussion. MTs have traditionally thought to be involved in the regulation of essential trace metals, such as copper and zinc and in the detoxification of essential and non-essential metals. More central functions are attributed to this proteins such as the intracellular scavenging of free radicals in protecting cells against oxidative stress (Chubatsu and Meneghini, 1993) and zinc-mediated gene regulation (Zeng et al., 1991a,b). The exposure of an organism to toxic factors, such as d^{10} metal ions, induces MT expression in different tissues. Therefore, these proteins could be used for exposure assessment in free-living organisms and in environmental pollution assessment.

Mammalian MTs have been widely studied and many of the proteins have been sequenced. However, so far, only the primary structure of a few MTs from aquatic invertebrate tissues has been elucidated, either by amino acid sequence analysis or by deduction from MT cDNAs or mRNAs. Among these are the MTs from crabs (Pedersen et al., 1994; Brouwer et al., 1989), mussel (Mackay et al., 1993; Khoo and Patel, 1999) snail (Berger et al., 1995), sea urchin (Wang et al., 1994) and oyster (Roesijadi et al., 1989).

Two dimensional NMR and X-ray crystallography have made considerable progress in the determination of the structure of MT in mammals and crabs. These studies revealed that in mammals, MT is a monomeric dumbbell shaped protein with seven metal ions located in two separate metal thiolate clusters. The metal ions in both clusters are tetrahedrally co-ordinated by both bridging the thiolate ligands. The three-metal cluster is located in the N-terminal β-domain and the four metal clusters in the C-terminal α-domain (Braun et al., 1992; Robbins et al., 1991; Dabrio et al., 2002). The other liquid phase NMR structures are those of equinoderme (Riek et al., 1999) and crab (Narula et al., 1995). Both structures revealed a monomeric protein composed of two globular domains binding a four- and a three-metal cluster in the equinodermal MT and two three metal-clusters in the crustacean MT.

Previous studies indicated that in the clam *Ruditapes decussatus*, a suspension-feeding bivalve mollusc widely distributed, MT was induced after exposure to Cd (Bebianno et al., 1993, 1994). However, the full characterisation of this protein was still unknown. Therefore, the aim of the present study was to characterise, identify, purify and sequence the NH_2-terminal of the MTs isoforms from the clam *R. decussatus*.

2. Materials and methods

2.1. Reagents and materials

Superdex 75 and Resource Q were purchased from Pharmacia LKB Biotechnology AB and Whatman®, respectively. Reverse-phase C18 microbore cartridges and reagents employed for N-terminal sequencing and HPLC analysis were purchased from Applied Biosystems®. PMSF, DTT and Trizma base were from Sigma Chemical Co. All other chemicals and reagents were from Merck or from Sigma Chemical Co.

2.2. Animals and cadmium exposure

R. decussatus (shell length 29–34 mm) were collected from Ria Formosa lagoon (South of Portugal) in October 1996 and maintained in 10 l tanks at 17 °C with aerated seawater contaminated with 100 μg Cd^{2+} l^{-1}, salinity 34‰, for 20 days. The water in the tank was changed three times a week and 200 clams were used for tissue preparation, MT purification and metal content.

2.3. Tissue preparation

Bivalves were frozen at −80 °C. The digestive part was dissected (45.14±0.01 g) and homogenised immediately (IKA, model Ultra-Turrax T-

25) in seven volumes of 20 mM Tris–HCl buffer (pH 8.6 with 0.1 mM PMSF and 0.5 mM DTT) (7 min, 4 °C), followed by centrifugation at 30 000 × *g* (1 h, 4 °C). The supernatant was treated at 80 °C for 13 min in an orbital shaker, to precipitate the high molecular weight proteins and centrifuged at 30 000 × *g* (1 h, 4 °C).

The final supernatant extract was concentrated by ultrafiltration (Amicon, YM1) and stored at −80 °C in 3 ml aliquots.

2.4. Metallothionein purification

Aliquots of the heat-treated homogenate were fractionated by gel-permeation chromatography (Superdex 75, Pharmacia). The elution was made with Tris–HCl 20 mM, pH 8.6 containing 150 mM NaCl, 0.1 mM PMSF and 0.5 mM DTT. For each fraction, the absorbance at 254 nm was measured and cadmium concentration analysed by graphite furnace atomic absorption spectrometry (Zeeman® Varian SpectrAA 300/400). The presence in the eluate of proteins with high sulfhydryl content was investigated further by differential pulse polarography (Methrom 646, model 646 SMDE) as described by Bebianno and Langston (1989). The column (26 mm × 60 cm) had been calibrated with the following markers of known molecular mass: blue dextran (2000 kDa), bovine albumin (67 kDa), ovalbumin (43 kDa), dessulfoferredoxin (*D. gigas*) (28 kDa), ribonuclease A (13.7 kDa), cytochrome *C* (12.4 kDa) and rubredoxin (6 kDa). Cadmium-enriched fractions containing material with molecular weight similar to rabbit MT I (Sigma) and with high sulfhydryl content, were pooled, concentrated and dialysed by ultrafiltration (Amicon, YM1). This pooled sample was fractionated further by ion exchange chromatography (Resource Q, Pharmacia) equilibrated with Tris–HCl 10 mM pH 8 as starting buffer, followed by an increasing step gradient from 10 to 800 mM (see Fig. 2). The eluate obtained was fractionated while the UV absorbance at 254 nm was monitored. The main cadmium binding fractions obtained were then pooled and concentrated (Amicon, YM1).

The proteins eluted from ion exchange chromatography were assayed for homogeneity by electrophoresis under denaturing conditions (SDS-PAGE) in mini-slab gels (70 × 80 × 1 mm). Stacking gels were 0.1% SDS, 5% polyacrylamide, and separating gels were 0.1% SDS and 17.5% polyacrylamide. Gels were stained by the Coomassie Blue G-250/H3PO4 protein staining method (Mitra et al., 1994) and by the silver-stain method (Blum et al., 1987).

2.5. Determination of metal content

The cadmium content was obtained by graphite furnace atomic absorption spectrometry (Zeeman® Varian SpectrAA 300/400).

2.6. Molecular weight estimation of the protein

The Superdex 75 column used for purification was calibrated with Molecular Weight markers, range 6–67 kDa (as described above) and the relative elution volumes used for molecular weight estimation of cadmium binding proteins (CdMTs) from the clam *R. decussatus*. For comparison, the samples were subject to 17.5% SDS-polyacrylamide gel electrophoresis (PAGE) calibrated with Molecular Weight markers, range 6.214–16.949 kDa (Pharmacia). To avoid the formation of disulfide bonds β-mercaptoethanol was used in the buffer.

2.7. NH_2-terminal amino acid sequence

The sequence of the desalted samples obtained from the ionic exchange purification was determined by subjecting the intact polypeptide chain to Edman degradation. Standard procedures recommended by the manufacturer were used for the analyses in an automatic Applied Biosystems (model 477A). All chemicals used were from Applied Biosystems and were prepared as recommended. The separation and identification of the amino acid phenylthiohydantoin derivatives was performed in an on-line HPLC reverse-phase C_{18} model 130A.

3. Results

3.1. Purification and characterisation of the metallothioneins

R. decussatus in the presence of 100 μg Cd^{2+} l^{-1} accumulates the metal preferentially in the cytosol. The chromatogram obtained after the separation on Superdex 75 of the clam heat-treated cytosolic extract (Fig. 1) shows that approximately 95% of total Cd eluted with a retention time similar to that of MT-1 from rabbit liver (Sigma) showing also high absorbance at 254 nm. The catalytic wave (at −1.54 V) of the thawed clam extract, obtained by differential pulse polarography, confirms also the existence of proteins containing a high sulphydryl residue content (data not shown) with similar characteristics to the MT catalytic wave previously described by Bebianno et al. (1993) for different tissues of this species. The apparent molecular weight of the protein, calculated after calibration of the column with standard molecular markers, was 13 700 Da. This CdMT fraction was further purified by ion-exchange chromatography and the elution profiles at 254 and 280 nm (Fig. 2) showed four peaks (CdMT-1, CdMT-2, CdMT-3 and CdMT-4). The homogeneity of these fractions was demonstrated by 17.5% SDS-PAGE. Fraction CdMT-1, CdMT-2 and CdMT-3 showed a single band with a R_f similar to the R_f of MT-1 from rabbit liver (Sigma, Fig. 3). Although some high molecular weight proteins were present, due probably to inefficient heat-denaturation procedure, all the samples were pure enough at this stage, to exhibit metal-binding properties consistent with the expected relationship between MT SH groups and metals.

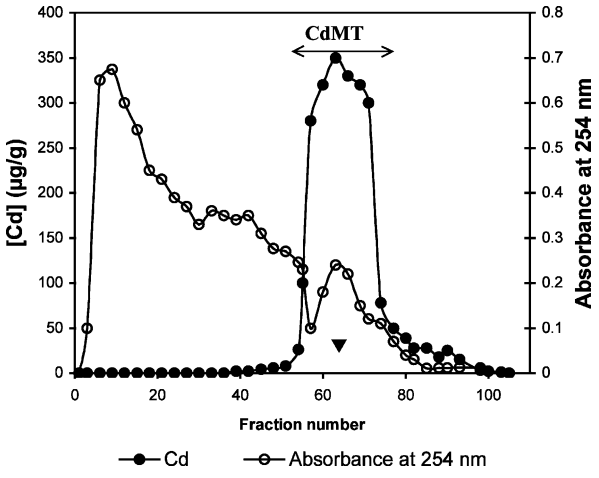

Fig. 1. Chromatographic Superdex 75 profile of heat-treated cytosol of cadmium exposed clams. The elution was made with 20 mM Tris–HCl, pH 8.6 (150 mM NaCl; 0.1 mM PMSF; 0.5 mM DTT) buffer. The double arrow shows the fractions pooled for further processing. The triangle shows the elution position of the MT standard.

The ultraviolet absorption spectra obtained for the four Cd^{2+}–MTs obtained from the Resource Q chromatography is characteristic of MTs (Kagi et al., 1974). With a high A_{254}/A_{280} ratio, a broad absorption envelope and a shoulder centred at about 254 nm typical of Cd–thiolate complexes (Vasak, 1991). The low aromatic content (absorption at 280 nm) was also evident (Fig. 2). On acidification (dissociation of the Cd–S bond) the absorbance at 254 nm disappeared when protons displace Cd, yielding the low pH spectrum of apo-MT. This result was confirmed for all four Cd^{2+}-binding proteins but is only presented for CdMT-1 in Fig. 4. The calibration of the PAGE with standard markers gave a molecular weight of 7.328 Da for all three fractions (CdMT-1, CdMT-2 and CdMT-3) but is only presented for Cd-BP1 in Fig. 5. Fraction CdMT-4 showed, in the same conditions, a faster electrophoretic mobility towards the anode. The molecular weight obtained by PAGE is significantly lower than that obtained for gel chromatography. The existence of disulfide bonds was ruled out because the electrophoretic behaviour of the protein was similar in the presence and absence of β-mercaptoethanol (results not shown).

Although the absorption coefficient of molluscs apo-MT could be higher once the amino acid content of these proteins are higher (72–75 amino acids) than mammalian ones, the protein concentration was calculated using the absorption coefficient of the mammalian (62 amino acids) apo-MT in 100 mM HCl at 220 nm ($\varepsilon = 48\,200\ M^{-1}\ cm^{-1}$), as described by Vasak (1991), because no absorption coefficient have been reported for molluscs apo-MT. Protein concentration along with the Cd content determined in each fraction and the

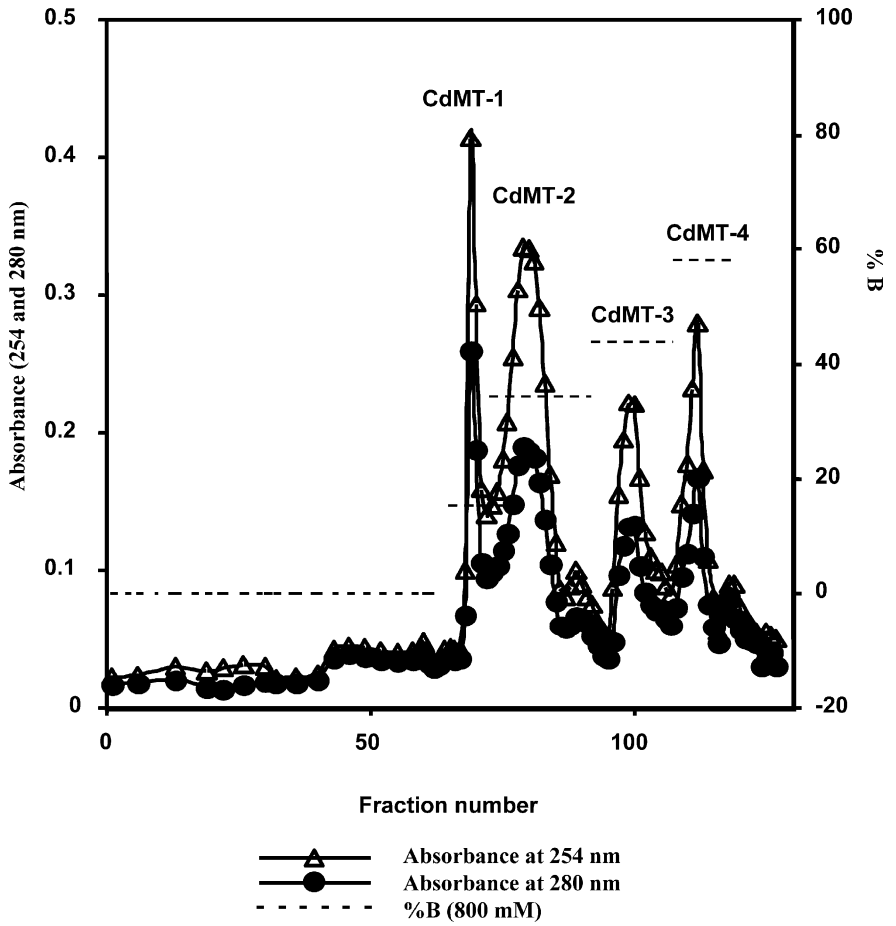

Fig. 2. Ion exchange chromatography of main cadmium-containing peak (CdMT) of Fig. 1 on Resource Q. Elution gradient was carried out as described in Section 2. The double arrow denotes effluent fractions pooled for further characterised. The dashed line indicate the increasing concentration of Tris–HCl buffer gradient from 10 to 800 mM.

stoichiometry of Cd^{2+} binding to *R. decussatus* MT are presented in Table 1.

After desalting and drying, the four samples were subject to an NH_2-terminal sequenciation. The CdMT-1 sequence was determined through residue 19 (Table 2). This region included glycine at the NH_2-terminal amino acid and two Cys-XY-Cys sequences considered characteristic of MT. CdMT-2 was only sequenced through residue 5 and is probably an isoform of the latter in which the aspartic acid was substituted by a glutamic acid. CdMT-3 was contaminated with sample CdMT-2 and CdMT-4 was blocked at the NH_2-terminal having a NG-sequence, a known phenomenon due to the cyclisation of ammonia.

Table 1
Stoichiometry of metal in Cd–MTs of the clam *R. decussatus*

	MT[a] (mol × 10^6)	Cd[b] (mol × 10^6)	Cd–MT
CdMT-1	0.02	0.160	8.0
CdMT-2	0.01	0.076	7.6
CdMT-3	0.01	0.062	6.2
CdMT-4	–[c]	0.054	–[c]

[a] Calculated from the absorption of the apoprotein (ε_{220} = 48 200 M^{-1} cm^{-1}) and the value of molecular weight calculated by calibration on gel SDS-PAGE (7328 Da).

[b] Calculated by furnace atomic absorption spectrophotometry.

[c] This value was not calculated because of the abnormal electrophoretic behaviour on SDS-PAGE (see text).

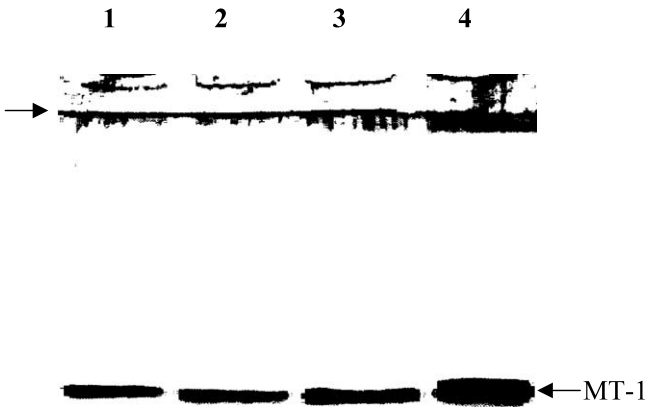

Fig. 3. SDS-PAGE of clam cadmium-binding proteins purified by ion-exchange chromatography (CdMT-1; CdMT-2; CdMT-3) and MT-1 from rabbit liver (Sigma). Stained by the silver-stain method as described in Blum et al. (1987). Lane-1, CdMT-1; Lane-2, CdMT-2; Lane-3, CdMT-3; Lane-4, MT-1 from rabbit liver (Sigma).

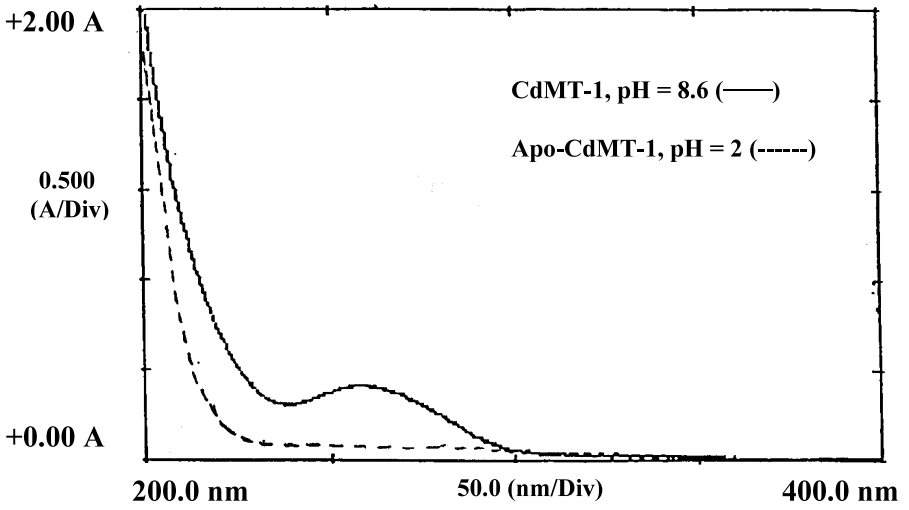

Fig. 4. Ultraviolet absorption spectra of one isoform of MT (CdMT-1) in the presence and absence of cadmium. CdMT-1 in Tris–HCl 10 mM, pH 8.6 (— —) and apo-MT in 100 mM HCl (---------).

4. Discussion

In many species MT occurs in several isoforms that differ substantially in amino acid composition. All mammals tissues examined thus far usually contain two major class I MT isoforms, designated as MT-I and MT-II (Kagi and Kojima, 1987a). So far, only a few sequences of molluscan MTs have been characterised in the mussels *Mytilus edulis* (Mackay et al., 1993), *Perna viridis* (Khoo and Patel, 1999) and the oyster *Crassostrea virginica* (Roesijadi et al., 1989; Unger et al., 1991).

In other marine organisms namely the mussel *M. edulis* (Mackay et al., 1993) two families of MT differing in size, were present with apparent molecular weight of around 10 and 20 kDa. Our results of gel permeation chromatography agree with those for the oyster *C. virginica* (Roesijadi et al., 1989) and the crab *Carcinus maenas* (Pedersen et al., 1994) who showed only a single peak at the 10–13 kDa position. In fact the results of Super-

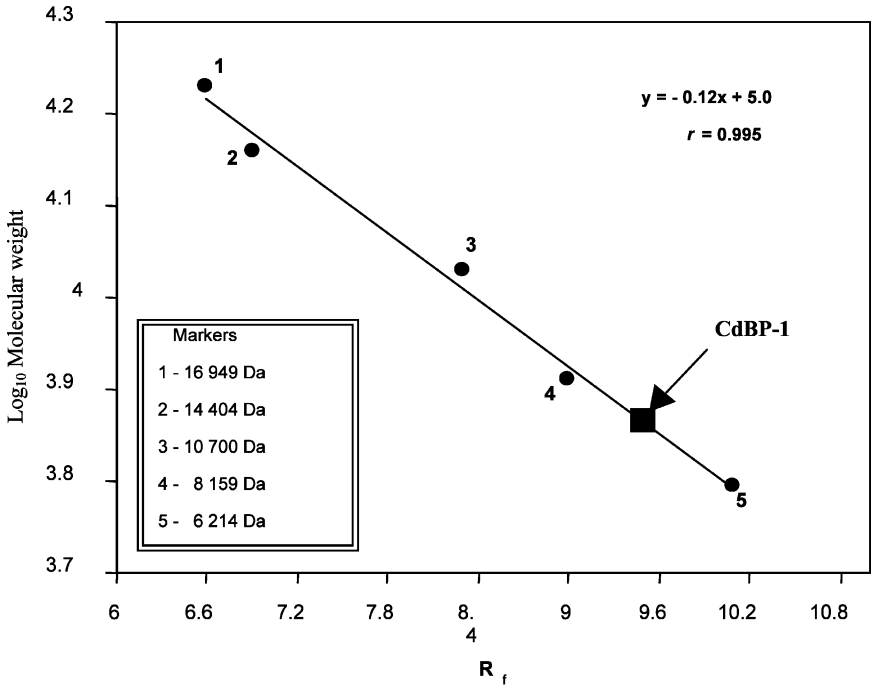

Fig. 5. Determination of molecular weight of clam MT by SDS gel electrophoresis calibrated with molecular weight markers ranging from 6214 to 16 949 kDa.

dex 75 (Fig. 1) demonstrate that all the cadmium was associated with a CdMT fraction with an average apparent molecular weight of 13 700 Da, which was different from the molecular weight of 7328 Da obtained on calibrated gel SDS-PAGE electrophoresis. This discrepancy is largely reported in well-characterised MT proteins from other organisms (Mackay et al., 1993; Olafson et al., 1979; Roesijadi et al., 1989), and is attributed to their flexible (Vasak et al., 1984), nonglobular, prolate ellipsoid shape with a calculated axial ratio of 6 (Kagi et al., 1974).

Cd–MTs from the clam *R. decussatus* was separated into four components (Fig. 2). All these components were purified. They all show to bind cadmium and have a characteristic absorption spectra (with low 280 nm absorption and a shoulder at 254 nm) consistent with the lack of aromatic residues in the amino acid composition (Fig. 4) and of Cd^{2+} thiolate co-ordination indicative of the presence of Cd–thiolate clusters (Vasak et al., 1984). This absorbance profile was similar to that reported for a Cd–MT by Bernhard et al. (1983). The exact nature of this electronic transition is still under discussion. The polarographic activity measured in the fractions with high cadmium content after Superdex 75, similarly agreed with the characteristic amino acid compo-

Table 2
NH_2-terminal amino acid sequence of *R. decussatus* cadmium binding proteins

	NH_2-terminal amino acid sequence
CdMT-1	GDPCNVAETGQCVCAQCCK————
CdMT-2	GEPCN————————————————————
CdMT-3	n.d.*
CdMT-4	n.d.**

sition indicating the presence of a high cysteine (thiol) content typical of MTs (Raspor et al., 1989) and confirming the results already obtained for this species by Bebianno et al. (1993).

Based on the chain molecular weight obtained by gel SDS-PAGE electrophoresis the three clam CdMTs isolated bind a total of six to eight cadmium atoms (Table 1). This results of stoichiometry metal/chain of MT confirm the existence of a cluster identical to the one suggested for others MTs isolated and structurally characterised (Coleman, 1993).

From the NH_2-terminal sequence analysis results (Table 2) only two isoforms in the digestive gland of *R. decussatus* were sequenced. It is possible that these isoforms arise due to polymorphism within the clam population as a large number of clams were required to prepare the MTs. Allelic polymorphism between clams is more likely due to the high degree of heterozygosity in invertebrates than in vertebrates. To date the highest number of isoforms found in aquatic species was in mussel *M. edulis* MT (at least nine Cd-induced isoforms, Mackay et al., 1993). The CdMT with a NH_2-terminal blocking moiety might also be a common feature in aquatic invertebrates. Similar results were obtained for one oyster *C. virginica* isoform (Roesijadi et al., 1989).

As already mentioned, the function of this protein is unclear and the reason for this large number of isoforms in marine invertebrates is also not clear. Suggestions included different specificity of the promoter region of the gene (Schmidt et al., 1985), different metal-binding affinities (Winge and Miklossy, 1982) or simply the need for a large number of copies to facilitate a rapid response when necessary (Olafson et al., 1979). Considering the NH_2-terminal sequence results (Table 2), the absence of methionine is consistent with other aquatic invertebrate MTs (Roesijadi et al., 1989; Mackay et al., 1993) indicating that MTs in this types of organisms are subject to NH_2-terminal modifications following initial synthesis that does not occur in the mammalian MTs.

The glycine content observed for the clams MTs are unusual for mammalian MT and the similarity in the NH_2-terminal of the clam MT with the

Table 3
Alignment of *R. decussatus* MT isoform (CdMT-1) with some representative class I MT

Crab-2[a]		P	D	P	C	C			N	D	K	C	D	C	K	E	G	E	C	K	T	G	C	K	C	T
Mouse MT-1[a]		D	P	N	C	S	C	S	T	G	G	S	C	T	C	T	S	S	C	A	C	K	N				
Trout MT-A[a]		M	D	P	C	E	C	S	K	T	G	S	C	N	C	G	G	S	C	K							
Oyster CdBP-1[a]	M	S	D	P	C	N	C	I	E	T	G	T	C	A	C	S	D	S	C	P	A	T					
Mussel MT-10-IV[b]		P	A	P	C	N	C	I	E	T	N	V	C	I	C	D	T	G	C	S	G	E	G	C	R	C	G
Mussel MT-20-I[b]		P	G	P	C	N	C	I	E	T	N	V	C	I	C	G	T	G	C	S	G	K	C	C	R	C	G
Clam (CdMT-1)		G	D	P	C	N	V	A	E	T	G	Q	C	V	C	A	Q	C	C	K							
Clam (CdMT-2)		G	E	P	C	N																					

[a] Roesijadi et al., 1989.
[b] Mackay et al., 1993.

Table 4
Comparison of MT NH_2-terminal sequence from *R. decussatus* with other MT sequences on the basis of optimised FASTA scores, identity and length of overlapping region using the FASTA sequence comparison program (Lipman et al., 1990)

Systematic group	Species	MT isoform	Optimised FASTA score	Identity (%)	Length of overlap (number of residues)	Reference
Invertebrates						
Mollusca	*C. gigas*	MT	97	77	17	[a]
	C. virginica	MT	93	59	17	Unger et al. (1991)
	M. edulis	MT10-IV	73	41	22	Mackay et al. (1993)
		MT20-I	77	41	22	Mackay et al. (1993)
	Littorina littorea	MT	62	47	17	[a]
	P. viridis	MT	73	50	16	Khoo and Patel (1999)
Crustacea	*Homarus americanus*	CuMT-I	55	43	16	Brouwer et al., (1989)
Vertebrates						
	Carassius auratus	MT	88	50	18	Chan (1994)
Fish	*Rutilus rutilus*	MT	90	50	18	[a]
	Danio rerio	MT	88	50	18	[a]
	Thermarces cerberus	MTA	85	44	18	[a]
	Barbatula barbatula	MT	86	44	18	Kille et al. (1991)
Bird	*Columba livia*	MT-1	62	46	15	Lin et al. (1990)

MT—metallotionein. Information was derived from a search of the SWISS-PROT data base.
[a] Sequences obtained from NCBI data bank with the accession numbers: CAC82788 for *C. gigas*, AAK56498 for *L. littorea*, P80593 for *R. rutilus*, NP 571150 for *D. rerio*, CAA65932 for *T. cerberus*.

oyster MT may support that this might be a molluscan variety of MT. The clam MT occurred in at least four isoforms whose only difference appear to be the presence in one of them of a blocked NH_2-terminal similarly to what has happened in the oyster *C. virginica* (Unger et al., 1991). In mussels glycine residues are also present in high amounts (around 15%) and do not have to have any specific sequence relationship with other amino acid and are apparently randomly distributed throughout the sequence (Mackay et al., 1993).

A comparison between *R. decussatus* NH_2-terminal MT with those of other species so far reported was made on the basis of optimised FASTA scores (Lipman et al., 1990) and showed a higher degree of similarity with the oyster MT NH_2-terminal (77% identity for *Crassostrea gigas* and 59% for *C. virginica*) than with the mussels one (41% for *M edulis* and 50% for *P. viridis*, Table 4). As a conclusion the clam MT sequence belongs to the class I MTs, like the other molluscan MTs and more recently to the family molluscs MT. Interestingly, molluscan MTs appear to be more closely related to vertebrate MTs than to those from other invertebrate phyla (Table 3; Unger et al., 1991; Dallinger et al., 1993).

A consensus alignment of clam MT NH_2-terminal sequence with some representative class I MTs, shown in Table 4, highlights the alignment of cysteine residues amongst this group of MTs. The percentage identity apart from the cysteine residues in this portion of the protein is relatively low. This is the most variable region of the mammalian molecules (Kagi and Kojima, 1987a,b) indicating few functional restraints on residues other than cysteine in this domain which is possible due to the lack of any recognisable secondary structure. In general, the most functionally important amino acids are conserved to a high degree.

5. Conclusions

It could be concluded that clam CdMT is a MT that belongs to the class I MT family and recently to the mollusc MT family. The positions of the cisteinyl residues with the oyster and mussels MT (Table 3) were highly conserved. Ion-exchange chromatography showed the presence of four low molecular weight cadmium-binding proteins in *R. decussatus*. Based on cadmium content and UV spectra, there are four different isoforms of *R. decussatus* MT but the results of NH_2-terminal sequence were conclusive in only two of them. There is also evidence in this species of polymorphism as previous reported for mussel MTs (Mackay et al., 1993) which can be a characteristic amongst invertebrate MTs.

Further investigation of the number and inducibility of the isoforms as well as metal binding characteristics will be necessary for the use of clam MTs as biomarkers.

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