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Cloning, expression and characterization of metallothionein from the Antarctic clam *Laternula elliptica*

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Abstract

The genes for two apparent subtypes of metallothionein (MT) isoform were isolated from the Antarctic clam *Laternula elliptica*. Determination of the nucleotide sequence showed that the gene consists of 222 bp that code a 73-amino acid protein. The comparison between MT cDNA sequences of *L. elliptica* and other bivalves showed strong homologies on positions of cysteine residues, which are important for their metal binding abilities. The gene for the MT was inserted into a pET vector and overexpressed as a carboxyl terminal extension of glutathionein-*S*-transferase (GST) in *Escherichia coli*. After the GST fusion proteins had been purified by glutathione–Sepharose affinity chromatography column and digested with enterokinase, the MT was purified with gel filtration and analyzed for its biochemical properties. Recombinant MTs were reconstituted with Cd, Cu, and Zn, and kinetic studies of the reactions with electrophilic disulphide, DTNB, were investigated to explore their metal binding ability. It is revealed that the Cd–MT and Zn–MT react with DTNB biphasically, and that Zn–MT reacts with DTNB more rapidly, and with a significantly greater pseudo-first-order rate constant. Cu–MT reacts monophasically and releases metal slowly from MT.

Keywords: Antarctic clam; Cadmium; *Laternula elliptica*; Metallothionein; Metal-binding ability

MTs are low molecular mass cytosolic proteins found in animals, higher plants, eukaryotic organisms, and some prokaryotes. MTs have a unique structure with a high cysteine content but contain few aromatic or histidine residues [1–3]. The predominant feature of MT is that one-third of its amino acids are cysteines, which are sulfydryl residues able to bind divalent metal such as Zn, Cu, and Cd. It also shows a conserved Cys-Xn-Cys motif, where X can be any amino acid other than cysteine. Most vertebrate MTs are made a single polypeptide chain and are comprised of two structural domains, designated α and β, which are capable of binding metals independently and are separated by a short linker region [4,5], although invertebrate MTs structure is still poorly understood. The biological functions of MTs are still a subject of controversy. Their induction by various metals implies an essential role in heavy metal detoxification, whereby they could facilitate the accumulation of toxic heavy metals, such as Cd, by chelating metal ions within the cell; however, the mechanisms for excretion of metal ions are not known [6–8]. MTs are also transcriptionally induced by various physiological and toxicological stimuli, such as oxidative stress, suggesting that *in vivo* they may neutralize hydroxyl radicals, cytokines, chemicals, and heat as well as heavy metals [9–13].

The MTs isolated from invertebrate species are much more variable in their cysteine residue alignment, domain, and central domain structure. Two isoforms with two cDNA sequences of MT were identified in the common mussel *Mytilus edulis* [14]. Several MTs have also been reported in a number of mollusk species including crab, oyster, snail and sea urchin [15–18]. However, the species studied to date represent a relatively small number of the

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invertebrates and provide only a narrow picture of the evolutionary diversity that may actually exist.

The clam *L. elliptica* shows widespread geographical distribution in the Antarctic region and represents an important species in coastal water ecosystems [19]. These mollusks are sessile filter feeders that can effectively concentrate seawater pollutants and, for this reason, they are used as sentinel organisms in biomonitoring programs. Considering the ability of many invertebrates to accumulate toxic heavy metals, induction of MTs in invertebrates is proposed as a specific biomarker response to metals. That is, an increase in MT synthesis is associated with increased capacity for binding these metals and, thus, increased resistance to metal toxicity [20]. Antarctic marine ecosystems show uniquely high levels of toxic heavy metals such as Cd in surface waters, accounted for by the upwelling of nutrient rich deep water [21]. Also, *L. elliptica* showed a naturally high accumulation of Cd in the body [22]. Currently, preliminary results report immuno-histochemical responses to an antibody of MTs in the gill, kidney, and digestive gland of Cd-exposed *L. elliptica*, indicating the presence of MTs induced by Cd [23]. Findings also revealed that MTs play an important role in Cd sequestration in the cytosol of *L. elliptica* kidneys and digestive glands. These results suggest that *L. elliptica* may have developed specific Cd detoxifying and controlling mechanisms to adapt to the environment [24].

Therefore, study of the structure and regulation of MT genes is important for a better understanding of both the physiological roles and the correct utilization of this metalloprotein. Although many studies have described MTs in coastal organisms in temperature environment, few data have been known about the MT genes in the Antarctic organisms [25,26]. The aims of the work described here were the identification of the coding sequences of MT genes in the Antarctic clam *L. elliptica*, and the comparison of these with previously reported MTs in coastal mollusks. We have also successfully expressed *L. elliptica* MTs in *Escherichia coli*. After purification and identification, the expressed proteins were analyzed to explore their metal-binding abilities.

Materials and methods

Cadmium exposure experiments and preparation of tissues

Laternula elliptica (shell length ≈ 80 mm) were hand-collected by SCUBA divers from 20 to 30 m depths in Marian cove near King Sejong Station (62°13′S, 58°47′W) in December 2001. After being acclimated to experimental conditions (ca. 1.0 °C) for 2 days, *L. elliptica* were exposed to a sublethal concentration of 50 ugCd/L for 8 days without feeding.

cDNA synthesis

Total RNA was extracted from the digestive gland of *L. elliptica* using Trizol reagent (Invitrogen Life Technologies, MD). Concentration of total RNA was determined by measuring UV absorbance at 260 nm. RNA purity was checked by determining the A_{260}/A_{280} ratio, and its integrity was checked by formaldehyde agarose gel electrophoresis. Complementary DNA (cDNA)[2] was synthesized using approximately 5 μg of total RNA. Oligo$(dT)_{20}$ primers were added and the final volume was adjusted to 20 μL with DEPC-treated water. This mixture was denatured at 70 °C for 5 min, and put on ice to allow the primers to anneal to the template. Samples were reverse-transcribed for 90 min at 42 °C with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RTR, Promega), RNAse inhibitor (RNAsin, Promega) and dNTPs in a reaction volume of 25 μL. The reaction was stopped by incubating for 5 min at 94 °C.

Polymerase chain reaction

The reverse-transcribed products were used for polymerase chain reaction (PCR) amplification performed with a set of sense and antisense primers corresponding to an open reading frame of MT-IA (GGATCCATGCCTGCACCTTGTAACTGTATCGA and CTCGAGTATTCACTTGCAGGAACAGCCAGGTG, respectively) designed from a known *Mytilus edulis* mRNA sequence (GeneBank Accession no. AJ005451). Polymerase chain reactions were performed using buffer, *Taq* polymerase (TAKARA), primers (10 pM each), cDNA template 1 μl, dNTPs 2.5 mM of each, and nuclease free water. All reactions were performed in a final volume of 20 μL. The thermal cycling program used to amplify the MT was performed on a thermocycler (TAKARA) and was configured as follows: 30 cycles of 45 s at 94 °C, 45 s at 48 °C, and 30 s at 72 °C followed by a single cycle at 72 °C for 5 min.

Cloning of MT cDNA and sequencing analysis

Purified PCR products were ligated into the pGEM-T easy vector system (Promega) with T4 DNA ligase. *E. coli* JM109 high-efficiency competent cells (Promega) were transformed with 10 μL of the ligation mixture and plated on ampicillin plates containing Xgal and IPTG. Insert-containing plasmids were purified using the plasmid purification kit (Bioneer). Individual colonies were screened by restriction enzyme analysis with *Bam*HI and *Xho*I followed by agarose gel electrophoresis. pGEM-T plasmids containing the correct size insert were sequenced on a fluorescent automated sequencer from both the 5′and 3′ends with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, USA). Characterization searching was performed using the BLAST program [27], using the nucleic acid and predicted amino acid sequences

[2] *Abbreviations used:* MT, metallothionein; GST, glutathionein-*S*-transferase; cDNA, Complementary DNA; IPTG, isopropyl-β-D-thiogalactopyranoside; DTNB, 5,5′-dithio-bis(2-nitrobenzoate).

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to compare the MT sequence to others deposited in multiple databases.

Construction of the expression vector for MTs

To construct the expression plasmid, we purified pET41a vector from a 100 ml bacterial culture and digested it with the cloning restriction enzymes (*Bam*HI and *Xho*I). The cloning plasmid (pGEM–MT1) had been purified from a similar volume of bacterial culture and digested with the same restriction enzymes. Then, the insert and vector were separated by an electrophoretic run on 1.5% agarose gel and extracted from the excised bands. Starting from 10 μg of both insert and vector, the ligation of cDNA with linearized pET41a vector was carried out using T4 DNA ligase with different vector/insert ratios. Ligation was transformed into *E. coli* JM109 competent cells, according to a standard protocol. The efficiency of ligation and transformation procedures were checked by plating on Luria–Bertani (LB, 0.5% yeast extract, 1% Bactotryptone, and 1% NaCl) agar plates containing 40 μg/ml kanamycin. More than 20 individual colonies were picked and inoculated in 10 ml LB broth for rapid screening by restriction map. Finally, the recombinant vector was checked for the correct orientation and cDNA sequence by sequencing in both directions. Recombinant pET41-MT plasmid was extracted, purified, and used to transform competent BL21 (DE3) cells, which permit high levels of expression. Selection of transformed colonies was performed on LB agar plates containing 40 μg/ml kanamycin. To identify the more efficient clones, a small-scale expression was carried out and the pET41-MT1 recombinants were screened for fusion protein expression on 15% SDS–PAGE.

Bacterial expression of recombinant MTs

Successfully transformed *E. coli* were picked from a single colony and grown overnight at 37 °C in LB medium, supplemented with 40 μg ml^{-1} kanamycin. The culture mixture was then inoculated to fresh LB medium (1:50 dilution) containing kanamycin and grown at 37 °C with continuous shaking, until the absorbance at 600 nm reached 0.6–0.8. To optimize culture conditions, recombinant MT expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the transformed *E. coli* and the bacteria were incubated at 30 or 37 °C for a period of 1, 2, 3, 4, 5, 6, or 20 h. The degree of expression was evaluated by SDS–PAGE.

Purification of recombinant protein and enterokinase cleavage

Prior to purification, the induced bacteria were harvested by centrifugation at 6000*g* for 20 min at 4 °C. The bacterial pellets were then resuspended in lyses buffer (20 mM Tris–HCl, pH 7.5, 0.2 mM PMSF, and 0.1% Triton X-100), and lysed by using lysozyme. The suspension was incubated at room temperature for 30 min with gentle shaking in order to thoroughly lyse cells. The lysate was further disrupted by sonication on ice until the sample was no longer viscous. Centrifugation was performed at 16,000*g* for 20 min to remove the insoluble cell debris and the supernatant was collected. At this point, the recombinant MT could be purified by affinity chromatography using glutathione–Sepharose 4B to selectively bind the GST tag of the fusion protein. The purified fusion proteins were digested by enterokinase at 25 °C for 4 h on an orbital shaker, and then applied to Sephacryl S-100 for the separation of the MT and GST. Peaks were detected by UV absorption. At each step of the purification procedure, we checked the presence of the recombinant MT on 15% SDS–PAGE, performed according to the classical method of Laemmli. Gels were stained with 0.1% Coomassie brilliant blue R-250 in 45% methanol and 10% acetic acid and properly destained. For higher sensitivity, MT was also visualized by silver staining performed according to a standard method. The amount of total protein recovered from each step of purification was evaluated by Bradford assay, using bovine serum albumin as standard. At the end of the purification process, we estimated the concentration of purified MT by measuring the absorbance of the metal-free protein at 220 nm in acid conditions using $\varepsilon_{220} = 47{,}300\ M^{-1}cm^{-1}$ [28]. As a standard for this assay, we employed commercial MT.

Preparation of Cd–MT, Zn–MT and Cu–MT

The reconstitutions of halo-MTs were carried out by the method of Vašák [29] to avoid oligomerization of the protein that can occur during the metal substitute process. For preparing Cd–MT, Zn–MT, and Cu–MT, an aliquot of the purified MT was acidified with HCl to pH 2.0 and chromatographed on a Sephacryl S-100 column equilibrated with 0.1 M HCl to remove the bound metal ions. Reconstitution with Cd, Zn, and Cu was achieved by the addition of 8.0 mole equivalents of Cd, Zn, and Cu followed by neutralization of the sample to pH 8.0 with 200 mM Tris, respectively. The unbound metals were removed by Chelex 100.

Reaction with DTNB

The competitive reactions with 5,5′-dithio-bis(2-nitrobenzoate) (DTNB) were carried out by a method previously described, with slight modifications [30]. In brief, 0.5 nmol of MTs were dissolved in 100 ml of 10 mM Tris–HCl buffer, pH 8.1, and placed in a quartz cuvette. The reaction was started by adding 1.5 mM of DTNB. The absorbance at 412 nm was recorded on the spectrophotometer for 100 min at 25 °C. As a blank. The same buffer containing 1.5 mM of DTNB ware used.

Results and discussion

Cloning of Laternula elliptica MT genes

The cDNA reverse transcribed from the *L. elliptica* mRNA was cloned into a TA cloning vector and analyzed

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L.elliptica MT10a    ATGCCTTCAC CTTGTAACTG CCGTGAAACC GGAAAATGCA CTTGTGACGG AAAGTGCTCG  60
L.elliptica MT10b    .......... .......... .......... .......... .......... ..........  60
M.galloprovincialis  ......G... .......... TATC...T.A AATGTG..T. TC....G.AC TGG...TAGC  60
D.polymorpha         ...AG.GAT. .A..C..... .GT....... ..TG.T...C G...C.C..A TGG.A...GC  60

L.elliptica MT10a    GGAGACGCGT GTTGCTGTGG TGCAAACTGT AACTGTGGAG AGGGCTGCAA GTGC--CCGG  118
L.elliptica MT10b    .......... .......... .......... .......... ...A...... ....--.A..  118
M.galloprovincialis  ..T..A.GT. ..C....... ..ACGC...C ..G..CTC-- -....GC.G. C..TAAAT.T  117
D.polymorpha         A.C...---. ...C.AACT. CAAGTGTG.C G..A.CT.TA ..T..A.... ACC.AA.T.T  117

L.elliptica MT10a    GGTG-CAAGA CTGTCGTCTG CAAATGCTCA GGCGACTGTG CCTGCGGAAA GGGTTGTACC  177
L.elliptica MT10b    ....-..... .......... .......... ........C. .......... ..........  177
M.galloprovincialis  TCC.GTTGT. AA..A..T.. ...G..T... ..TAG..... .G..T.A.GC A..G.....A  177
D.polymorpha         T...G..... A...GACA.. ......TGG. .AAA.....C AG.....CGT ...A..C..T  177

L.elliptica MT10a    GGACCCGATT CCTGCAAGTG TGATGCTGGA TGTTCCTGCA AGTGA 222
L.elliptica MT10b    .......... .......... .......... .......... ..... 222
M.galloprovincialis  ..G..TTCAA .G..T.GA.. ..CAC....T ..C....... .A... 222
D.polymorpha         ..G..A.... .G....CA.. ...CAGC..C ..C..T.... ..... 222
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Fig. 1. The nucleotide sequences of *L. elliptica* MT 10a and MT 10b cDNA were aligned with those of other mussels using ClustalW 1.83 [37].

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L.elliptica MT10a    MPSPCNCRET GKCTCDGK-C SGDA-CCCGA NCNC-GEGCK CPGCKTVVCK  47
L.elliptica MT10b    .......... .........-. ....-..... ....-..D.. .Q.......  47
M.galloprovincialis  ..A....I.S NV.I.GTG-. ..EG-.R..D A.K.S.AD.. .S...-....  47
D.polymorpha         .SD....V.. .D.R.ADGS. .DCSN.K..D S.K.SKPN.- .G--.N.T..  47

L.elliptica MT10a    CSGDCACGKG CTGPDSCKCD AGCSCK 73
L.elliptica MT10b    .......... .......... ...... 73
M.galloprovincialis  ...S...EA. ....ST.R.A P..... 73
D.polymorpha         .GEN.Q..V. .......T.. S..... 73
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Fig. 2. Protein sequences of *L. elliptica* MT10a and MT10b were aligned with those of other mussels using ClustalW 1.83 [37].

for its sequences (Fig. 1). After sub-cloning, the 222 bp of two different MT cDNA fragments were confirmed by sequencing (GenBank Accession Nos. DQ832722, DQ832723). These sequences displayed only three different nucleotide points, and encoded two different protein sequences. Each fragments were sequenced on both strands and manual verification of the chromatograms corroborated the difference in the sequences. These were translated in 73 amino acids, of which 22 were cysteine residues (Fig. 2). The two protein sequences differed by replacement of Asp for Gly in position 35 and by Gln for Pro in position 39. The comparison between MT-10 cDNA of *L. elliptica* and known MTs of other bivalves reveal highly conserved cysteine positions. Moreover, the cysteines are organized in terms of nine CXC motif arrangements, and these clusters are mainly involved in the capture of the metal ions. Usually, vertebrate MTs contain 61–62 amino acid residues, whereas larger chains with 72–74 amino acid residues are found in mollusks and in nematodes [3]. Also mollusk MTs have a high glycine content (≈15% in mussels) randomly distributed throughout the sequence [31,32]. As expected, we found that *L. elliptica* MTs have high glycine content, but they also contained an elevated number of lysine residues not found in mussels. The high number of lysine residues in *L. elliptica* MTs (11% vs. 6.8% in mussels) may explain the decreased ability of the protein to release metals. The decreased mobility of cadmium in MTs may be due to a stronger metal–thiolate interaction because of the increased number of lysines. Pan et al. [33] observed that a substitution of three lysines with glutamates in the CK motifs of MT modified the metal-binding ability of MTs [33]. They suggested that the lysine residues of MTs are not critical for maintaining protein structure, but that they play a role in regulating the microenvironment and stability of both metal-binding clusters.

Expression and purification of MTs

To introduce the amplified MT 10a clone into the pET41 expression vector, the vector was digested with *Bam*HI and *Xho*I and the linear DNA was eluted from the gel. The MT clone was digested by the same restriction enzymes and a DNA fragment of 240 bp was eluted. The resulting fragment was ligated to pET41 and transferred into *E. coli* JM109. The transformants were grown in LB medium supplemented with kanamycin (40 μg/mL) and the plasmid DNA was isolated. The putative clones were screened primarily by size selection, digested with *Bam*HI and *Xho*I, and finally confirmed by DNA sequencing. The resulting clone was named the pET–MT1 plasmid and transferred into *E. coli* BL21 (DE3) for expression of GST–MT1. The expression vector pET–MT1 contains the coding region for the MT 10a, and its expression can be induced by adding IPTG accumulates, high amounts of a soluble GST–MT

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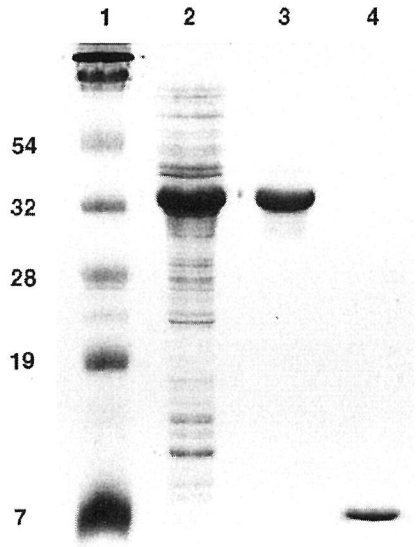

Fig. 3. Identification of the supernatent of the *E. Coli* cell lysate on 15% SDS–PAGE: lane 1, protein standard; lane 2, supernatent; lane 3, purified metallothionein after GSTrap chromatography; lane 4, MTs in pooled and concentrated fraction after protease cleavage.

fusion protein migrating in SDS–PAGE with an apparent molecular weight of 30 kDa (Fig. 3, lane 2). The overexpressed protein was not detected in an uninduced extract (data not shown). Expression was then performed on a large scale in LB culture. The pellet recovered from 1 L culture (about 3 g wet weight) was resuspended in PBS buffer, and lysed by using an ultrasonic cell disruptor. After cell lysis, GST–MT1 fusion protein was purified by GSTrap FF affinity chromatography. Recombinant MT was separated from the GST-tail by enzymatic cleavage using enterokinase, which recognizes and selectively cuts a sequence located immediately upstream from the multiple cloning site. The digestion products of GST fusion protein were applied to Sephacryl S-100 for the separation of MT and free GST. The free recombinant MT presents a molecular weight of 7 kDa (Fig. 3, lane 4), and the yield of the purified MT was 5 mg/l of culture.

Kinetic studies with DTNB

The DTNB reactions were carried out under pseudo-first-order conditions (excess DTNB) at 25 °C and pH 8.0. The reaction of Zn–MT, Cd–MT, and Cu–MT with DTNB went to completion within 100 min. The first-order plots for the reactions of the three MTs with DTNB were obtained by plotting ln $(A_\infty - A_t)$ vs time (Fig. 4). Both the reactions of Zn–MT and Cd–MT showed biphasic kinetics with fast and slow steps. The two observed rate constants (k_f and k_s) could be calculated according to the standard kinetic treatments for parallel reactions, where 'f' and 's' denoted fast and slow step, respectively. The reaction of Cu–MT exhibited a monophasic kinetic process. The observed rate constant of Cu–MT was two and six times slower than the value of the fast reactions of Cd–MT and Zn–MT, respectively, and similar to those obtained in the slow step reaction (Table 1). Munoz et al. reported the reactivities of the isolated α and β domains of Cd–MT with DTNB for comparison with that of the haloprotein [34]. Their results showed biphasic kinetic behavior in which the kinetics of

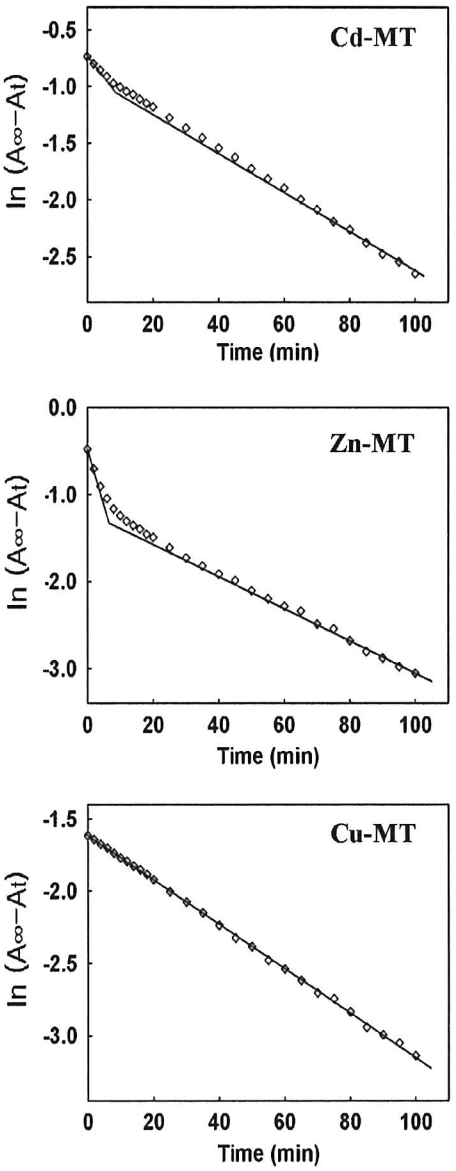

Fig. 4. Kinetic reaction of MTs with DTNB.

Table 1
Rate constants for the reactions of MTs with DTNB

Reactants	Fast step components ($k_f \times 10^4/s^{-1}$)	Slow step components ($k_s \times 10^4/s^{-1}$)
Zn–MT	15.74	3.28
Cd–MT	4.87	3.03
Cu–MT	2.55	

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DTNB reaction with the α-domain are monophasic with the fast step of the observed reaction rate, and the β-domain is confirmed as the site of the kinetically slow step.

Relevant information about the 3-D structure of MTs allowed us to clarify the structural features supporting the observed differences in reactivity. MT structures from invertebrates show a monomeric protein composed of two globular domains that possess a dumb-bell-like shape with uniformly sized and almost spherical C-terminal α- and N-terminal β-domains. The metal ions in both clusters are tetrahedrally coordinated by both terminal and bridging thiolate ligands, and the two protein domains are connected by a flexible hinge region in the middle of the polypeptide chain [5,35]. In other studies, the binding affinities of the two domains to divalent ions showed that the ^{111}Cd-intramolecular exchange rates for β-domains are approximately 2000 times as fast as those for α-domains, while the α-domain has a greater affinity for Cd [3]. Another NMR study of Cd–MT for intermolecular metal displacement reactions demonstrated that the two domains react independently of one another, and that the ^{111}Cd chemical shifts of the Cd^{2+} ions in the α-domain are completely unaffected by the binding of other metal to the β-domains. Our kinetic studies of Cd–MT and Zn–MT showed biphasic reactions, suggesting that they are apparently decomposed into component reactions that sum to yield the overall reaction. The fast step occurs exclusively in the β-domain, and the slow step predominantly in the α-domain.

One of the key questions concerning the postulated functions of MTs in detoxification and essential metal metabolism is that of the role of its two structurally distinct metal-thiolate clusters, located in well-separated protein domains. The differential reactivity and metal-binding affinity of the two clusters of MTs have suggested that the two domains play multiple cellular roles [36]. For example, the α-domain binds toxic metal ions such as Cd, whereas the β-domain participates in the essential metal metabolism of Zn used for homeostatic purposes. Although all MTs are suggested to have similar roles in metal-detoxification and metal-resistance, the properties and mechanisms of these proteins are still to be explored.

In this paper, we have established the cDNA sequence for two isoforms of MTs from the Antarctic clam *L. elliptica*. We also analyzed the metal-binding abilities of recombinant MTs with DTNB competition reactions, which suggested that the α and β domains of MTs might have different metal reactivities, implying different functional adaptations to the variant environment. Further investigation will be needed to study the structure/reactivity relationships between the metal-binding abilities of the two structurally different domains of MTs.

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