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Freezing and anoxia stresses induce expression of metallothionein in the foot muscle and hepatopancreas of the marine gastropod *Littorina littorea*

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Summary

Differential screening of cDNA libraries constructed from the foot muscle of marine snails *Littorina littorea* revealed several cDNAs that are upregulated during anoxia or freezing exposures, environmental stresses that are naturally endured by this species. One full-length clone of 1196 nucleotides (GenBank accession number AY034179) hybridized with a 1200-nucleotide band on northern blots and encoded a 100-amino-acid protein that was identified as belonging to the metallothionein (MT) family. *L. littorea* MT shared 45% and 56% identity with the copper- and cadmium-binding MT isoforms, respectively, from another gastropod, *Helix pomatia* and 43–47% identity with marine bivalve MTs. The *L. littorea* sequence included the mollusc-specific C-terminal motif Cys-X-Cys-X(3)-Cys-Thr-Gly-X(3)-Cys-X-Cys-X(3)-Cys-X-Cys-Lys that identifies it as a family 2 (mollusc) MT. Northern blot analysis showed that *L. littorea* MT was upregulated in both foot muscle and hepatopancreas in response to both freezing and anoxia stresses; within 1 h of the beginning of the stress transcript levels rose 2.5- to sixfold of control levels, reaching maximal levels at 12 or 24 h. After 24 h recovery from either stress, transcript levels were reduced again in three cases but remained elevated in hepatopancreas from anoxia-treated snails. Upregulation of MT during environmental stress could serve one or more possible roles, including a function in antioxidant defense.

Key words: environmental stress, gene expression, metallothionein, invertebrate, anaerobiosis, freeze tolerance, periwinkle, *Littorina littorea.*

Introduction

Species that inhabit the intertidal zone possess a high degree of metabolic plasticity that enables them to tolerate the constantly changing environmental conditions imposed by the tidal cycle. One such species, the periwinkle snail *Littorina littorea*, is highly tolerant of oxygen deprivation and has also developed the ability to survive freezing. Many of the biochemical mechanisms that support freezing and/or anoxia survival in intertidal invertebrates have been identified. Anaerobiosis is supported by large stores of fermentable fuels (glycogen, aspartate) in all tissues and the use of modified pathways of substrate catabolism that link additional sites for the substrate-level phosphorylation of ADP with the production of end-products, including alanine and succinate (De Zwaan, 1983; Storey and Storey, 1990; Storey, 1992). Furthermore, as residual oxygen is depleted from tissues, anoxia-tolerant marine molluscs sharply lower their metabolic rate to <10% of the resting aerobic rate at the same temperature, thereby greatly extending the time that their fixed reserves of metabolic fuels can support anaerobiosis. Periwinkles also have a well-developed ability to endure the freezing of extracellular body fluids. In fact, survival after 8 days frozen at –8°C has been documented (Murphy, 1983). Freeze tolerance allows *L. littorea* to colonize the intertidal zone in temperate and subarctic regions and to endure low tide exposure to winter air temperatures that can fall well below the freezing point of body fluids (approximately –1.9°C for snails in full-strength seawater).

In recent studies, our laboratory has begun to document the gene-expression responses involved in natural stress tolerance in *L. littorea*. Larade et al. (2001) reported transcriptional upregulation of the ribosomal protein L26 during anoxia, and proposed that upregulation of the protein might function to stabilize the existing mRNA pool during the anoxic period until normal oxygen levels are resumed. More recently, Larade and Storey (2002) described a novel cDNA that is both transcribed and translated during anoxia exposure, supporting a role in anoxia survival. In the present study, we report the isolation of a metallothionein (MT) cDNA that is upregulated in response to both anoxia and freezing exposures in *L. littorea*.

MTs are a family of low molecular mass, cysteine-rich proteins that characteristically bind both essential and non-essential metal ions in a metal/sulfur complex. Although all

MTs show similar functional properties and are uniformly found in species ranging from microorganisms to mammals, they are notoriously variable in terms of amino acid composition and sequence. Despite this, there are enough similarities among primary structures to permit a classification scheme comprising 15 families, as defined by Binz and Kagi (1999) (see also http://www.expasy.ch/cgi-bin/lists?metallo.txt). MTs are commonly composed of 58–75 amino acids, approximately 30% of which are cysteine residues and few, if any, are aromatics. Characteristically, the cysteines are arranged in repeating Cys-X-Cys motifs throughout the sequence. Although a definitive picture of their cellular function has yet to unfold, the metal-binding characteristic confers several roles to MTs, including: (1) homeostasis of essential trace metals, (2) segregation of toxic heavy metals (e.g. cadmium, mercury, lead), and (3) a reservoir for copper and zinc, which can then be donated to other metalloproteins such as transcription factors. Studies have also produced several lines of evidence to indicate that MTs have antioxidant properties, either directly, due to their many, readily oxidized cysteine residues, or indirectly, by binding heavy metals ions (e.g. copper). This latter action prevents metal ions from participating in the Fenton reaction, which is a primary source of highly reactive hydroxyl radicals in cells (Viarengo et al., 1999).

MTs have been sequenced from several mollusc species (Unger et al., 1991; Dallinger et al., 1993, 1997; Mackay et al., 1993) and MT protein levels in tissues of marine molluscs rise in response to heavy metal challenge (especially cadmium) (Langston and Zhou, 1986, 1987; Langston et al., 1989; Boutet et al., 2002). However, the present study is the first to clone and sequence the MT cDNA from a marine gastropod and, more importantly, the first to show that MT expression is induced in response to naturally occurring environmental stresses. Here we report the upregulation of MT expression in *L. littorea* during anoxia or freezing exposures, and propose several mechanisms by which MT may contribute to stress tolerance.

Materials and methods

Animals: collection and treatment

Marine periwinkles *Littorina littorea* L., were collected in Nova Scotia, Canada and purchased from a local supplier. Snails were held for 3 weeks in the laboratory at 5°C in full-strength seawater (1000 mosmol l^{-1}) before use. Control snails were sampled from this condition. Shells were quickly cracked open, foot and hepatopancreas were immediately removed, frozen in liquid nitrogen, and stored at –80°C until use. To impose anoxia, other snails were placed in closed jars with 1–2 cm of seawater. The water was previously bubbled with 100% nitrogen gas for at least 10 min and this was continued for 15–20 min after adding snails to the jar. This procedure normally results in a reduction in O_2 content to <1% of normal values. After testing to confirm the absence of O_2, the jars were sealed and maintained under anoxic conditions at 5°C for 1, 12 or 24 h (each group in a separate jar). Snails from a separate group were exposed to anoxia for 6 days and then returned to aerobic conditions by transferring them to a jar with normoxic water and air at 5°C and sampled after 24 h of recovery. For freezing exposure, snails were removed from aerated seawater, placed in closed plastic containers lined with damp paper towelling, and transferred to an incubator set at –8.0°C. Preliminary tests were conducted with several snails to determine the rate of cooling and length of time before ice nucleation occurred. A thermistor placed inside the shell in contact with the mantle showed that nucleation occurred within 45 min, so experimental freezing exposures of 1, 12 or 24 h were timed after that length of prefreeze cooling. Other snails were frozen at –8.0°C for 6 days, and then returned to aerated seawater at 5°C for 24 h before sampling. Initial tests showed virtually 100% survival of this freezing protocol.

cDNA cloning, screening and sequence analysis

Screening of the anoxic library

RNA isolations, cDNA library construction, screening and northern blot analysis were all performed essentially as described previously (Cai and Storey, 1996). Poly(A)$^+$ mRNA was isolated from the foot muscle of snails exposed to 1, 12 or 24 h of anoxia. Equal amounts of mRNA from each time point were pooled (totalling 5 μg) and then used to construct a cDNA library in the LambdaZap vector, which was then subjected to two rounds of differential screening. Plaques (~2.5×10^4 pfu 24 cm^{-2} plate) were grown on lawns of *Escherichia coli* (XL1 blue) and transferred to duplicate nylon membranes (Hybond N, Amersham Biosciences, Baie d'Urfe, Quebec, Canada). These were hybridized at 42°C for 16 h with ^{32}P-labelled single-stranded cDNA probes synthesized from the poly(A)$^+$ RNA prepared from foot muscle of aerobic (control) *versus* anoxic snails. The anoxic probe was also synthesized using a composite pool of mRNA (equal amounts from 1, 12 and 24 h anoxic snails). Plaque lifts were washed at room temperature with low stringency (2× SSC, 0.2% w/v sodium dodecyl sulfate) to high stringency (0.2× SSC, 0.2% w/v sodium dodecyl sulfate) washes (SSC, 150 mmol l^{-1} NaCl, 15 mmol l^{-1} sodium citrate). Plaques showing a higher signal with the anoxic *versus* aerobic probe were purified with a second round of plaque hybridization. Duplicate lifts were concurrently hybridized, washed and exposed to film. The purified clones in pBluescript plasmid vectors were rescued by *in vivo* excision using ExAssist helper phage (Stratagene, La Jolla, CA, USA). Plasmids were digested with *Eco*RI and *Xho*I to release the cDNA insert.

Screening of the freeze library

A cDNA library was prepared from foot muscle of freeze-exposed snails in the same manner as for the anoxic library but using equal amounts of poly(A)$^+$ mRNA isolated from snails frozen for 1, 12 and 24 h. Screening was conducted with ^{32}P-labelled probes made from mRNA isolated from control (aerobic, unfrozen) *versus* frozen snails (synthesized from a composite pool of mRNA from 1, 12 and 24 h frozen snails).

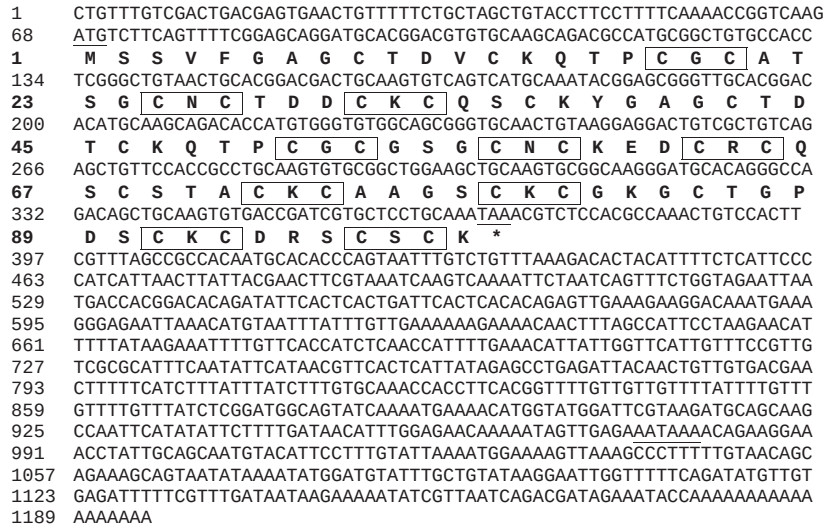

Fig. 1. cDNA sequence and translated amino acid sequence of *L. littorea* metallothionein, LL_{MET}. Underlined nucleotides show the start codon, the stop codon (asterisk) and the polyadenylation signal (AATAAA), respectively. The encoded amino acid sequence is shown in bold. Note the high percentage of cysteine residues, most of which are arranged in a repeating Cys-X-Cys pattern.

Northern blot analysis

Total RNA was isolated from organs of control, anoxic or frozen snails using TRIzol™ (Gibco Life Technologies, Carlsbad, CA, USA), and separated on formaldehyde–agarose gels using 16 μg of total RNA per lane. After transfer to HybondN membrane by capillary action, the quality of total RNA was assessed by the identification of a well-defined ribosomal band (stained with ethidium bromide). The LL_{MET} probe was synthesized by random primer labelling of the clone insert (Sambrook et al., 1989). Blots were hybridized overnight with labelled probe (10^7 c.p.m. ml^{-1} hybridization solution) at 42°C, followed by low (2× SSC) and high (0.2× SSC) stringency washes until there was a strong contrast between the signal and background, and then exposed to film. Transcript levels were quantified by scanning the X-ray autoradiogram using a Scan Jet3C scanner (Hewlett Packard, Mississauga, Ontario, Canada). The image was acquired using DeskScan (v2.2) software in conjunction with Imagequant V3.22 to quantify pixel density (Amersham Biosciences). The ribosomal bands were quantified and these values were used to evaluate any differences in loading between lanes. RNA transcript sizes were estimated based on comparison with the migration of standards in an RNA ladder (Gibco Life Technologies).

DNA sequencing and analysis

The clone was sequenced in both directions by Canadian Molecular Research Services (Ottawa, Ontario) and Bio S&T (Lachine, Quebec, Canada), using an automated sequencing procedure. Clone identification was attempted *via* sequence homology searches as determined with the NCBI blastn and blastx programs. Sequence analyses (alignments, comparisons, translations) were completed using DNAman vI (Lynnon BioSoft, Vaudreuil, Quebec, Canada). Percentage identities between *L. littorea* MT and other mollusc MT sequences were calculated from individual pairwise alignments as described in DNAman. A homology tree of invertebrate MT sequences was generated with sequences obtained from GenBank using the protein alignment matrix PAM (Dayhoff et al., 1978) as employed in DNAman.

Results

cDNA and amino acid sequence analysis

A cDNA representing a putative metallothionein (GenBank accession number AY034179) was isolated *via* differential screening of *L. littorea* foot muscle cDNA libraries. The same clone was isolated from independent screenings of two different libraries (freeze-treated and anoxia-treated snails), indicating that expression is increased in response to both stresses. The 1196 nucleotide (nt) clone, denoted LL_{MET}, represents a complete transcript and contains 3′ and 5′ untranslated regions (UTRs), a complete open reading frame (ORF) and polyadenylation signal (nt 975–980). In contrast to the short length of the 5′-untranslated segment (69 nt), the 3′-untranslated region extended 620 nt before the polyadenylation signal. This is much longer than the corresponding UTRs of the mammalian and *Drosophila melanogaster* MT sequences but is comparable to that of the sea urchin *Strongylocentrotus purpuratus* and lobster *Homarus americanus* (Harlow et al., 1989; Valls et al., 2001). A blastn homology search of GenBank using the *L. littorea* cDNA sequence as a query did not retrieve significant matches, so the translated ORF was used to classify the sequence.

At 100 amino acids in length and a predicted molecular mass of 10 kDa, the protein encoded by LL_{MET} (Ll-MT; Fig. 1) is significantly larger than most other MTs, regardless of species. Ll-MT also contained a correspondingly higher number of cysteine residues (27), 20 of which fall into the Cys-X-Cys motif pattern that is characteristic of all MTs. As with other mollusc MTs, Ll-MT lacks the additional Cys-Cys pattern found in crustacean, vertebrate, nematode and echinoderm MTs. Fig. 2A shows a comparison of the Ll-MT amino acid sequence with those of MTs from three other mollusc species: the copper-binding (Dallinger et al., 1997) and cadmium-

A

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Periwinkle Ll-MT  MSSVFGAGCTDVCKQTPCGCATSGCNCTDDCKCQS  35
Land snail Cu     MSGRGKN-----------------------------   7
Land snail Cd     MSGKGKGE----------------------------   8
Mussel (20-Ib)    -PGPCNCIETN-------------------------   7
Oyster            MSDPCNCIETG-------------------------   8

Periwinkle Ll-MT  CKYGAGCTDTCKQTPCGCGSGCNCKEDCRCQSCST  69
Land snail Cu     ------CGGACNSNPCSCGNDCKCGAGCNCDRCSS  35
Land snail Cd     -----KCTSACRSEPCQCGSKCQCGEGCTCAACKT  37
Mussel (20-Ib)    ---VCICGTGCSGKCCRCGDACKCASGCGCSGCKV  42
Oyster            ---TCACSDSCPATGCKCGPGCKCGDDCKCAGCKV  42

Periwinkle Ll-MT  ACKCAA-GSCKCGKGCTGPDSCKCDRSCSCK  100
Land snail Cu     -CHCSN-DDCKCGSQCTGSGSCKCGSACGCK   65
Land snail Cd     -CNCTS-DGCKCGKECTGPDSCKCGSSCSCK   67
Mussel (20-Ib)    VCKCS--GTCKCGCDCTGPTNCKCESGCSCK   72
Oyster            KCSCTSEGGCKCGEKCTGPATCKCGSGCSCK   75
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B

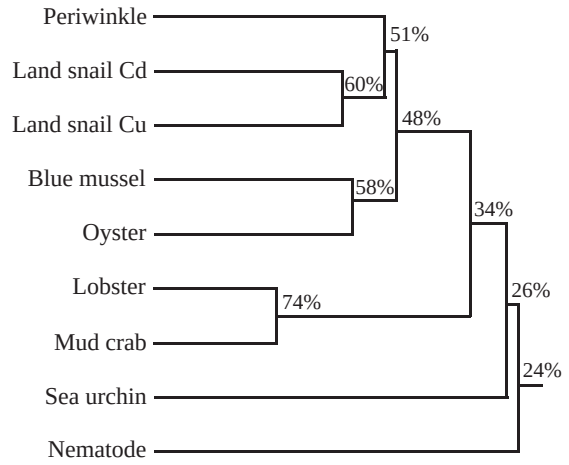

Fig. 2. (A) Translated amino acid sequence of clone LL_{MET} (Periwinkle, encoding the protein Ll-MT) aligned with amino acid sequences of metallothioneins (MT) from other mollusc species: land snail *Helix pomatia* cadmium (Cd) MT (midgut-derived, GenBank accession number P33187) and copper (Cu) MT (accession number P55947); blue mussel *Mytilus edulis* 20-Ib (accession number S47576) and oyster *Crassostrea virginica* (accession number S17156). Conserved amino acids are highlighted in black. Gray highlights indicate residues that are shared between Ll-MT and at least two other sequences in the figure. The boxed residues at the C terminus comprise a highly conserved motif, Cys-X-Cys-X(3)-Cys-Thr-Gly-X(3)-Cys-X-Cys-X(3)-Cys-X-Cys-Lys, which categorizes Ll-MT into the mollusc family (family 2) of the MT classification system. (–) indicates an inserted gap. (B) Homology tree of MTs from selected invertebrate species. The percentage identities between the groups are indicated to the right of the forks. Sequences were aligned and the homology tree generated using the protein alignment matrix (PAM; Dayhoff et al., 1978) as employed in DNAman. The sequences were obtained from GenBank; accession numbers for species not listed in A are: lobster *Homarus americanus* (accession number A37039), mud crab *Scylla serrata* (accession number P02805), sea urchin *Strongylocentrotus purpuratus* (accession number P04734) and nematode *Caenorhabditis elegans* (accession number A34905).

binding (Dallinger et al., 1993) MTs from the land snail *Helix pomatia*; isoform MT20-Ib from the blue mussel *Mytilus edulis* (Mackay et al., 1993) and MT from the eastern oyster *Crassostrea virginica* (Unger et al., 1991). Although these sequences are quite variable, the alignment of the cysteine residues is conserved. All 18 of the cysteine residues of the land snail MTs (both the copper- and cadmium-binding isoforms) were aligned with those of the marine snail *L. littorea*, albeit with the insertion of selected spaces. The oyster and blue mussel sequences have more cysteine residues (21 and 23, respectively), but 18 of them also align with those shared by the *L. littorea* and *H. pomatia* sequences. Fig. 2A also shows the presence of the conserved C-terminal sequence pattern, Cys-X-Cys-X(3)-Cys-Thr-Gly-X(3)-Cys-X-Cys-X(3)-Cys-X-Cys-Lys (Binz and Kagi, 1999). This motif is characteristic of mollusc MTs and its presence in Ll-MT confirms that the *L. littorea* protein is a family 2 (mollusc) metallothionein. Overall, Ll-MT showed the strongest sequence identity with the proteins from *H. pomatia* (56% identical to the cadmium-induced isoform, 45% identical to the copper-specific) as well as 47% and 43% identity with oyster and blue mussel MTs.

Metallothionein homology relationship

To further support the identity of the *L. littorea* clone as encoding a metallothionein, a homology tree was generated from a multiple alignment of Ll-MT and eight other invertebrate metallothionein sequences (Fig. 2B). Ll-MT is most similar to the other gastropod sequences, sharing 51% identity with the *H. pomatia* sequences, and sequences from gastropod molluscs are distinctly different from those of bivalve MTs. The crustacean sequences share the highest percentage identity (74%) but are distinct from the mollusc MTs. Both nematode and sea urchin MTs show very little sequence similarity with the molluscan proteins.

Northern blot analysis

The expression of LL_{MET} during freezing or anoxia exposure of *L. littorea* was assessed by northern blot hybridization using radiolabeled probe synthesized from the digested insert of the LL_{MET} plasmid. LL_{MET} expression in both foot muscle and hepatopancreas was analyzed in control snails at 5°C, snails subjected to 1, 12 or 24 h of stress exposure (either anoxia under an N_2 gas atmosphere at 5°C or freezing at –8°C), and snails given 24 h of recovery at 5°C after 6 days of stress. Although LL_{MET} was isolated from cDNA libraries synthesized from foot muscle mRNA, northern blot hybridization revealed that the transcript was also present in the hepatopancreas, an expression pattern that is consistent with the broad distribution of MTs among the tissues of these and other molluscs. For example, MT protein has been documented in the gill, kidney and digestive gland of *L. littorea* (Bebianno and Langston, 1998) as well as the foot and adductor muscles of the mussel *Mytilus galloprovincialis* (Carpene et al., 1983).

The LL_{MET} probe hybridized with a 1.2 kb band and the pattern of stress-induced transcript accumulation was similar

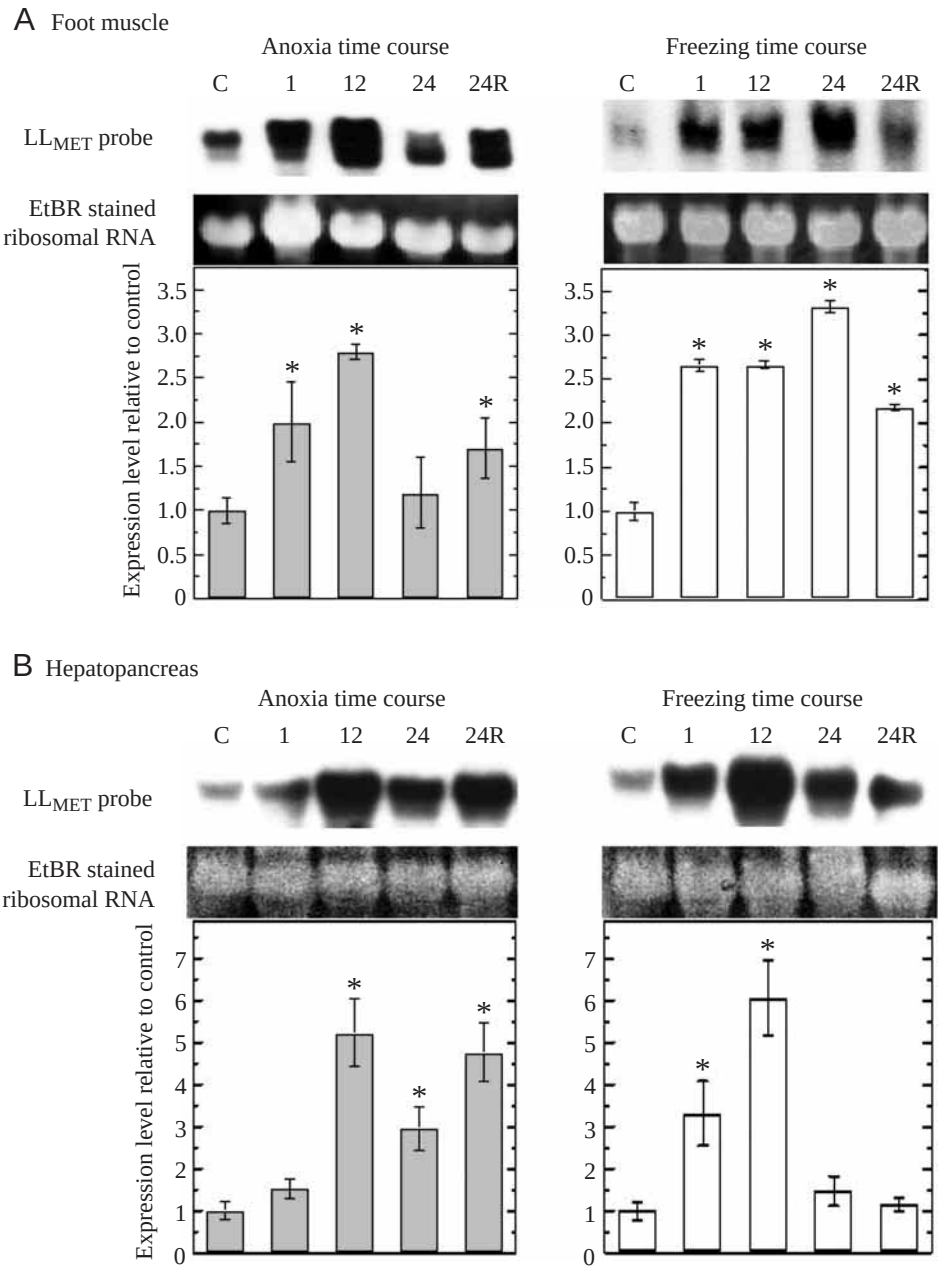

Fig. 3. Northern blot hybridization of clone LL_{MET} against total RNA (15 μg per lane) isolated from foot muscle (A) or hepatopancreas (B) of *L. littorea* showing changes in transcript levels over a time course of freezing or anoxia stress. Samples were collected from control (C, untreated), stressed (1, 12 or 24 h of anoxia or freezing exposure) and 24 h recovered (after 6 days of stress exposure) snails. Representative northern blots (top) are shown with the corresponding ethidium bromide-stained ribosomal RNA bands (middle) from the formaldehyde gel. Histograms (bottom) show normalized band intensities. Values are means ± S.E.M. for N=3 independent trials using RNA isolated from different individuals. Measured pixel densities for each band were first normalized against their corresponding rRNA band and then mean band intensity was calculated for each treatment. Finally, experimental values were plotted relative to the corresponding control value that was set to 1. Asterisks indicate values that differ significantly from controls, Student's t-test (P<0.05).

under both freezing and anoxia stresses. In foot muscle (Fig. 3A), LL_{MET} transcripts were present in low amounts in control snails but, during anoxia, transcript levels increased significantly (P<0.05) by twofold after only 1 h of anoxia exposure and further rose to a maximum of 2.8-fold higher than control values after 12 h. LL_{MET} transcripts declined to near-control values by 24 h of anoxia exposure, but recovery under normoxic conditions induced another significant increase to 1.7-fold that of control animals. The response to freezing in the foot muscle was a significant 2.6-fold increase in transcript levels within 1 h of freezing at –8°C with a continuing increase to reach a 3.3-fold maximum level after 24 h of freezing. After thawing for 24 h, transcript levels had decreased slightly but were still 2.2-fold greater than control levels. The effects of anoxia and freezing exposure on LL_{MET} transcript levels in hepatopancreas are shown in Fig. 3B. Transcript levels did not rise as quickly as in foot muscle (after 1 h levels were not significantly increased) but after 12 h of anoxia transcript levels peaked at a value that was much greater than in muscle, 5.2-fold higher than control values. As for foot muscle, this level of expression declined after 24 h to a value threefold higher than control levels, followed by a slight increase to 4.7-fold higher than controls after 24 h of normoxic recovery. The response to freezing was somewhat faster in hepatopancreas; transcript levels rose significantly by 3.3-fold within 1 h and reached a maximum expression level of sixfold greater than control values by 12 h. Transcripts fell sharply after freezing at –8°C for 24 h and, in contrast to the response during

recovery from anoxia, there was no secondary increase in transcript levels during thawing recovery.

Discussion

The marine periwinkle *Littorina littorea* is an ideal model system for the study of natural stress tolerance. In the intertidal zone snails encounter multiple environmental stresses including extremes of high and low temperatures, both hyperoxic and hypoxic conditions, and hyper- and hypo-osmotic stresses. As part of a search for gene expression responses that support stress tolerance, the present study shows that upregulation of metallothionein gene expression is a response to both anoxia and freezing stresses in periwinkles.

Since the 1970s, the concentrations of heavy metals in the tissues of marine molluscs have been used as a bioindicator of the level of pollution in the marine environment (Phillips, 1977). More recently, researchers have focused on MT protein levels as an indicator of heavy metal content in tissues because exposure to metals such as Cd, Zn and Hg seems to be a universal inducer of MT (Hamza-Chaffai et al., 2000; Boutet et al., 2002; Ceratto et al., 2002). This correlation has led several laboratories to pursue the cloning of molluscan MT gene sequences in order to elucidate the controls on MT gene expression and understand how MT protein levels are regulated *in vivo* (Tanguy et al., 2001; Ceratto et al., 2002). As a result, the MT gene family is relatively well described in marine invertebrates. In this study, we report the isolation of a metallothionein cDNA, denoted LL_{MET}, from the foot muscle of marine periwinkles *L. littorea*, analyze the Ll-MT protein sequence encoded by it, and illustrate the pattern of gene expression in response to anoxia and freezing stresses.

The catalogue of characterized MTs covers a range of species including vertebrates, molluscs, crustaceans, echinoderms and nematodes. All the proteins are similar in terms of structure and function but, apart from the conserved patterns of cysteine residues (C-X-C), show significant diversity in nucleotide and amino acid sequences. Vertebrate MTs are uniformly characterized by a low molecular mass (6–7 kDa), with 60–68 amino acids, 20 of which are cysteine residues and none are aromatic. By contrast, MTs from marine invertebrates also have a high cysteine content, but may also contain a few aromatic residues. Invertebrate MTs also display a broader range of molecular mass, but with an unusually long N-terminal sequence, Ll-MT is still 25–38 residues longer than nearly all other invertebrate MTs reported to date. A recent study by Park et al. (2002), however, described a MT containing 103 amino acids in the Asian periwinkle *Littorina brevicula*, so a MT sequence of ~100 amino acids may be characteristic of this group of gastropods. Unfortunately, the study by Park et al. (2002) presented only a short C-terminal sequence (21 residues) covering the highly conserved mollusc-specific motif (boxed region in Fig. 2A), so no comparison of the two species can be made with respect to the novel N terminus that was found in Ll-Mt. One unusual feature of MT in both littorine species, however, was the presence of an aromatic phenylalanine residue that is not found in other molluscs. Interestingly, Langston and Zhou (1986) reported the presence of a 10 kDa cadmium-binding protein in *L. littorea*. They showed that cadmium in *L. littorea* from unpolluted sites was primarily complexed with a metal-binding protein of approximately 20 kDa, but during exposure to high cadmium, a second binding protein of 10 kDa was induced, predominantly in the digestive gland. Based on the equivalent molecular masses, the currently identified Ll-MT protein may be the 10 kDa protein reported in this earlier work. Together with the current information, these data suggest that *Littorina* sp. may harbour a novel subclass of mollusc MTs.

Several amino acid motifs characterize the metallothionein family, the most prevalent being the presence of a large number of cysteine residues found in repeats of Cys-X-Cys. Although the MT sequences aligned in Fig. 2A are all from molluscs, the percentage identities are quite low. Despite this, 18 of the cysteine residues align perfectly, with 14 in the predicted pattern. The defining characteristic of the mollusc MT family is the presence of a conserved C-terminal sequence that is unique to this group [Cys-X-Cys-X(3)-Cys-Thr-Gly-X(3)-Cys-X-Cys-X(3)-Cys-X-Cys-Lys; Binz and Kagi, 1999]. As illustrated in Fig. 2A, this motif was found in Ll-MT, confirming it is a metallothionein of the mollusc class.

A unique biological feature of MT genes is their promiscuous expression in response to a variety of agents and conditions. Depending on the system, *de novo* synthesis of the protein can be induced by exposure to selected metals, cytokines, tumour promoters, hormones and growth factors (reviewed in Coyle et al., 2002) as well as by environmental stresses, including hypoxia (Murphy et al., 1999). To our knowledge, this paper is the only study to date to show enhanced MT gene expression in response to natural environmental stresses in a stress-tolerant animal. Northern blot analysis showed a three- to sixfold increase in MT mRNA levels in response to both anoxia and freezing. The induction response was essentially the same for both stresses, rising quickly and remaining high through at least 12 h of stress exposure. Such a pattern indicates that a common mechanism or trigger is responsible for activating the transcription of the MT gene, most likely low oxygen tension. Anoxia is, by definition, a lack of oxygen, whereas freezing creates an ischemic state where oxygen delivery to tissues is interrupted due to hemolymph freezing. It is understandable, therefore, that genes that are anoxia-induced could also respond to freezing stress.

Possible functions of MT during anoxia and freezing

Despite their initial discovery as metal-binding proteins, MTs are clearly multifunctional, and their roles may vary, not only in a species-specific but stress-specific manner. A particularly appealing function is one of an antioxidant, for which there is ample supportive evidence. Campagne et al. (1999) demonstrated that transgenic mice overexpressing the MT-1 isoform were protected against focal cerebral ischemia and reperfusion injury. In their study, the mRNA levels for

MT1 increased 7.5-fold over baseline values upon application of ischemia, and even though tissues were still damaged, the sizes of the affected regions were approximately 40% smaller. In a reverse study, Lazo et al. (1995) showed an enhanced sensitivity to oxidative stress in cultured embryonic cells that lacked genes for MT-I and II. A cold-induced MT response in the whole animal has also been reported in the literature, although not in a stress-tolerant species; Beattie et al. (1996) found that MT was induced in response to cold in rats that were transferred from 26°C to 6°C. Following an increase in mRNA content, the MT protein levels increased 1.4- to 3-fold in kidney and liver, whereas the thermogenic organ, brown adipose tissue, showed a 16-fold increase in MT protein. Thermogenesis is a process that requires high oxygen consumption and is also accompanied by a sharp rise in reactive oxygen species (ROS) generation. This led the researchers to conclude that MT was produced to provide antioxidant defense.

In invertebrates, additional supporting evidence for an antioxidant role for MTs comes from studies of the effects of iron and cadmium on the intertidal mussel *Mytilus galloprovincialis*. Because of its role in the Fenton reaction, exposure to high levels of iron stimulates ROS production whereas cadmium exposure does not. However, cadmium exposure did induce *de novo* synthesis of MT protein and Viarengo et al. (1999) showed that pre-exposure to cadmium greatly increased the survival rate of mussels that were subsequently exposed to iron in an anoxic environment. The protective effect was attributed to the cadmium-dependent induction of MT, thus supporting a role for MT in antioxidant defense. Interestingly, this antioxidant effect of MT does not appear to due to iron binding, as MTs do not bind iron particularly well. Rather, there is evidence that MT may be an inherent antioxidant, scavenging ROS *via* thiolate oxidation of the cysteine residues. Using cell-free systems, Thornalley and Vâsàk (1985) demonstrated that MT could quench hydroxyl radicals, whereas Irato et al. (2001) showed the same result could be achieved with living cells challenged with superoxide. Therefore, it is interesting to note that Ll-MT contains 27 cysteine residues, 4–7 more than MTs from previously described species, and these additional thiol-containing cysteine residues may allow for a stronger antioxidant activity in this stress-tolerant species.

Injury caused by reactive oxygen species (ROS) is a major source of cellular damage for organisms exposed to environmental stress. *L. littorea* can be viewed as a model for reperfusion injury because long bouts of anoxia or freezing are followed by a sudden reintroduction of oxygen. Such rapid changes in oxygen availability can cause a burst of ROS production sufficient to overwhelm the existing capacity of antioxidant defenses, thereby leading to oxidative damage. Studies with land snails have shown that antioxidant defenses are elevated when animals enter a dormant state (aestivation), even though this state is associated with reduced oxygen consumption (Hermes-Lima et al., 1998). It was hypothesized that the rise in antioxidant defenses during aestivation was a preparatory measure for combating oxidative stress when metabolic rate rose rapidly during arousal. A similar response might occur in *L. littorea*, antioxidant defenses being upgraded as a response to anoxia or freezing stress in order to limit the potential for damage from a burst of ROS production occurring as soon as oxygen is reintroduced. The rapid upregulation of MT gene expression documented here would support this idea as well as provide a way to sequester ions, such as copper, that can also catalyze the Fenton reaction. Interestingly, anoxia exposure of *L. littorea* also induces a rapid increase in transcript and protein levels of ferritin, an iron-binding protein (K. Larade and K. B. Storey, manuscript submitted for publication) as well as changes in the activities of some antioxidant enzymes and a strong increase in reduced glutathione content (Pannunzio and Storey, 1998). Hence, MT expression in response to anoxia or freezing stresses may be part of a concerted elevation of antioxidant defenses under these conditions.

Conclusion

Whole animal exposure to freezing and anoxia induced transcription of metallothionein in the tissues of *Littorina littorea*. The ability of MT to function as an antioxidant and as a reservoir of essential metals could contribute to survival under these stresses. The present results, in demonstrating MT induction in response to anoxia and freezing stresses, also suggest the need for caution in the use of MT protein levels as an index of the level of heavy metal pollution in marine ecosystem. Clearly, MT is inducible by multiple environmental stresses (anoxia, freezing, heavy metals), so the use of MT as a marker for heavy metal pollution would only be valid if 'marker-species' are not simultaneously exposed to other stresses. This could create a problem for the interpretation of long-term (year-round) studies, because seasonal changes in exposure to environmental low oxygen and low temperature could confound a correlation between MT and heavy metal levels.

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