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The basis for increased metallothionein in a natural population of *Crassostrea virginica*

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Metallothionein (MT) is elevated in a natural population of the oyster *Crassostrea virginica* that is exposed to cadmium. However, induction of MT is not a likely cause for this elevation. This was concluded after re-examination of the results of a study in which MT mRNA and MTs were analysed in oysters collected from a cadmium-contaminated environment. MT mRNA concentrations did not differ in oysters collected from a cadmium exposure gradient and could not account for increases in MT, although laboratory experiments had clearly shown that induction of MT in response to cadmium is seen at both transcriptional and translational levels. It appears that, at the low concentrations of cadmium encountered in natural environments even when contaminated, the underlying basis for the elevation of MT is its stabilization by cadmium and its accretion while being synthesized at basal rates, rather than induction of MT *per se*. This observation has implications for the efficacy of using either MT mRNA or MT levels in molluscs in assessing metal exposure or its biological effects in contaminated natural environments.

Keywords: metallothionein, mollusc, oyster, cadmium, metal.

Introduction

Putative functions for metallothionein (MT) include metal detoxification, zinc and copper homeostasis, scavenging free radicals, and involvement in the acute phase response (Cherian and Chan 1993). Specific cellular interactions for MT have yet to be definitively identified. However, studies on biochemical structure (Kägi 1993), molecular regulation (Radtke *et al*. 1993, Heuchel *et al*. 1994, Dalton *et al*. 1997, Bittel *et al*. 1998, Günes *et al*. 1998), and metal exchange between MT and other cellular macromolecules such as haemocyanin and various zinc metalloenzymes and transcription factors (Brouwer and Brouwer-Hoexum 1992, Maret 1994, Cano-Gauci and Sarkar 1996, Maret *et al*. 1997, Jacob *et al*. 1998, Roesijadi *et al*. 1998) have provided insights to the potential role of MT in the cellular processes noted above.

MTs have been identified in most major animal taxa (see for example Roesijadi 1992). Because of the central role that environmental toxicological considerations have played in studies on interactions between metals and aquatic organisms, the emphasis on MTs in this group is focused mainly on metal detoxification or on its use as a 'biomarker' in environmental monitoring for biological effects of metals. The conceptual justification lies in the fact that proteins characterized as MTs in aquatic organisms exhibit the basic characteristics of high metal content, induction by metals, accumulation during metal exposure, and correlation between overexpression and resistance to metal toxicity. Furthermore, mice with disrupted MT genes exhibit reduced resistance to metal toxicity (Michalska and Choo 1993, Masters *et al*. 1994), suggesting a direct relationship between MT and metal resistance that has been generalized to other animal species. Nevertheless, it should

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be recognized that the use of MT induction or increases in MT concentration as practical measures in environmental monitoring is based on still unproved assumption regarding their significance. The controversy over the function of MT in metal detoxification (Vallee 1995, Maret and Vallee 1998) serves as the underlying dynamic in any endeavour associated with its use in environmental monitoring.

Molluscs, mainly bivalves and gastropods, have frequently been used as biomonitors for environmental pollution, and this taxonomic group has been a focus for the study of MT in aquatic invertebrate species. Although detailed biochemical studies of molluscan MTs are still scarce, amino acid sequences deposited in protein sequence databases such as GenBank, EMBL, and Swiss-Prot are available for MTs from the oyster *Crassostrea virginica* (Unger *et al*. 1991), blue mussel *Mytilus edulis* (Mackay *et al*. 1993, Barsyte *et al*. 1999), zebra mussel *Dreissena polymorpha* (unpublished), and terrestrial gastropods *Helix pomatia* (Dallinger *et al*. 1993, Berger *et al*. 1997) and *Arianta arbustorum* (Berger *et al*. 1995). Amino acid sequences for the oyster, blue mussel, and zebra mussel MTs have been deduced from cDNAs whose sequences are also in the sequence databases. For these species, biochemical or molecular response of relatively well characterized MTs can be analysed at protein isoform and/or transcript levels.

A re-examination of the results of several studies conducted in this laboratory using the oyster *Crassostrea virginica* suggests that the notion of protein induction may not explain the detection of elevated concentration of MT in natural populations, although laboratory studies have demonstrated the intrinsic capability for metal-dependent induction of MT for this species. Accretion of MT that is synthesized at basal rates may be a more likely explanation. The basis for such conclusions are presented below.

Results and discussion

It is known from laboratory experiments that MT expression in the oyster *Crassostrea virginica* is time and metal-concentration dependent (Roesijadi 1996); that MT induction is accompanied by increased binding of metal ions and a redistribution of metal ions to MT (Roesijadi and Klerks 1989); that disruption of normal cotranslational processing occurs at high cadmium concentrations, resulting in an unexpected N-terminal modification of MT (Roesijadi *et al*. 1989, 1991); and that the last is associated with cadmium toxicity (Roesijadi *et al*. 1995, 1996).

It is clear that increases in MT concentrations in laboratory experiments are due to induction, since they are associated with increases in the responsible mRNA (Unger and Roesijadi 1996) and elevated rates of ^{35}S-cysteine incorporation by the protein (Roesijadi *et al*. 1991). That this is the case in oysters collected from a cadmium-contaminated estuarine environment adjacent to Chesapeake Bay is less clear. Cadmium concentrations in this region range from 0.0002 to 0.0006 μM (F. Riedel, Benedict Estuarine Research Laboratory, personal communication), the higher value representing a relatively high degree of contamination for a natural environment. Oysters in this environment possess tissue cadmium concentrations that increase linearly as the sites of collection approach an apparent upstream source (table 1). Although elevated, these concentrations are considerably lower than those in individuals exposed to cadmium in the laboratory, however. MTs are

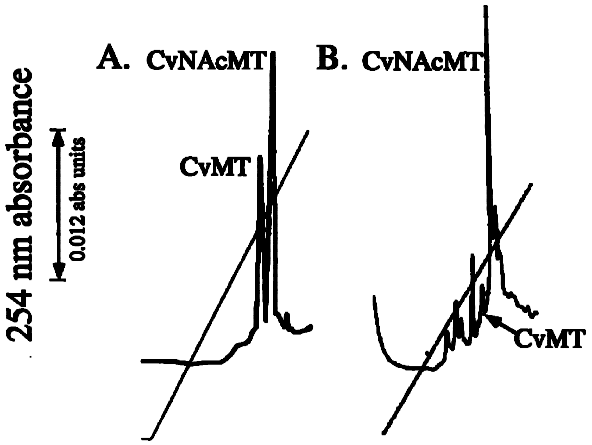

Figure 1. Anion exchange chromatograms showing (A) the elution profile of CvNAcMT and CvMT, the expected and unexpected forms of metallothionein from gills of *Crassostrea virginica* exposed continuously to 50 μg l^{-1} for 2 days (from Roesijadi *et al.* 1991) and (B) the elution profile showing mainly CvNAcMT in a representative sample of gill tissue from an oyster from the Patuxent River (from Roesijadi 1994). To date, CvMT has only been observed at significant levels in oysters exposed to relatively high concentrations of cadmium in the laboratory. CvNAcMT is the form observed under basal conditions and carries the expected N-terminal modification for proteins with the encoded N-terminal amino acid sequence (Roesijadi *et al.* 1991, Unger *et al.* 1991). It is induced in the laboratory and increases in concentration in a cadmium-contaminated environment.

elevated as well (Roesijadi 1994) and are mainly the expected N-acetylated form (figure 1). The total cadmium bound to these MTs increases linearly with the cytosolic, rather than total, cadmium concentrations, and there is a substitution of cadmium for zinc on MT molecules as the cadmium content of MT increases (Roesijadi 1994). On average about 24% of the accumulated cadmium is bound to MT (table 1).

From this information one can easily conclude that MT in this natural population is induced and exhibits certain relationships with the subcellular distribution of bioaccumulated cadmium. Interestingly, however, there are no significant differences in MT mRNA levels in relation to exposure to cadmium (table 1), although the cadmium and MT concentrations are elevated. The rate of MT synthesis is proportional to the levels of MT and mRNA in higher animals (Durnam and Palmiter 1981) and there is no valid reason to reject this basic notion in oysters. This is supported by a pulse-labelling experiment showing increased ^{35}S-cysteine incorporation into MTs in oysters exposed to cadmium in the laboratory (Roesijadi *et al.* 1991) under conditions in which the responsible mRNA is also elevated (Unger and Roesijadi 1996). Similar pulse-labelling of MT in oysters collected from the sites in which both cadmium and MT are elevated do not show corresponding elevations in radiolabelled MT (Roesijadi, unpublished data). Additionally, induction of MT in this species is accompanied by the appearance of a non-N-acetylated form of MT that is not expected (figure 1). Its appearance coincides with the first appearance of induced MT mRNA (Roesijadi *et al.* 1995, 1996) and is initially undetectable or present at concentrations lower than the expected N-acetylated form. It appears that induction by cadmium results in rates of MT synthesis that exceed the ability of cells to properly modify the N-terminal structure during cotranslational processing (Roesijadi *et al.* 1991). That this form

Table 1. Cadmium, percent of cadmium bound to metallothionein, and metallothionein mRNA concentrations in gills of *Crassostrea virginica* from the Patuxent River, Maryland (USA), tabulated as a function of location of collection site on a cadmium contamination gradient. Cadmium concentrations were determined by atomic absorption spectrometry of tissues or MT fractions (Roesijadi 1994); RNA concentrations were determined in RNase protection assays (Fuentes *et al.* 1994). Data were abstracted from previously published reports (Fuentes *et al.* 1994, Roesijadi 1994) and reorganized here, emphasizing the effect of collection site.[a]

Nautical miles upstream from river mouth	nmol Cd g^{-1} tissue[b], mean + 1 SE (*n*)	Percent of total Cd bound to MT[c,d], mean + 1 SE (*n*)	attomol MT mRNA g^{-1} tissue[d], mean + 1 SE (*n*)
5.7	17.8 ± 1.6 (30)	25.0 ± 5.6 (8)	38.2 ± 5.6 (20)
9.8	26.6 ± 1.4 (40)	29.0 ± 3.7 (19)	30.3 ± 3.9 (19)
15.7	42.9 ± 2.4 (40)	21.4 ± 2.3 (19)	42.8 ± 7.8 (19)
18.3	47.7 ± 2.6 (40)	20.1 ± 2.7 (13)	30.0 ± 4.2 (15)

[a] The table shows the site component of the results of previous two-way analysis of variance of data obtained as a site times date factorial analysis for tissue cadmium and MT mRNA (Fuentes *et al.* 1994, Roesijadi 1994). Percent of total Cd bound to MT was analysed by one-way analysis of variance.

[b] Significant at $p < 0.0001$.

[c] Individual values for MT-bound Cd were normalized to the mean total tissue cadmium concentration of each sample. Aberrant values for MT-bound cadmium at the upstream-most site (see Roesijadi 1994) were not used in these calculations.

[d] Not significant, $p > 0.05$.

of MT is not present at appreciable levels in oysters from the Patuxent River also argues against induction of MT in this environment. The lowest concentration of cadmium found to induce MT mRNA in our laboratory experiments is 0.004 μM, which results in only a slight increase in MT mRNA after 21 days (Unger and Roesijadi 1996). This concentration is greater than ten-fold that of the highest estimate of 0.0006 μM for this environment. Factors other than increased MT synthesis probably account for the observed increases in MT concentrations in light of a lack of evidence for transcriptional and translational induction of MT in this natural population of oysters.

The accretion of MT is dependent on the rate of turnover, which is defined by the rates of synthesis and degradation. It is well known that MT is rendered more refractory to proteolytic degradation upon binding cadmium (Winge and Miklossy 1982) and that the rate of turnover of Cd-MT is slower than for Zn-MT or Cu-MT (Webb 1987). The resistance of the cadmium-bound oyster MT to proteolytic degradation was verified in this laboratory when procedures to cleave the proteins with trypsin were being developed (Roesijadi *et al.* 1989). Thus, it is not unreasonable to suspect that the substitution of cadmium for zinc and a decreased turnover of Cd-MT may be a basis for the elevated levels of MT seen in this natural population of oysters. This brings into question both terminology and mechanistic assumptions related to increases in MT reported in studies of natural populations of molluscs. It seems that increases in MT in the population of oysters described above are due to factors other than induction. At the normally low concentrations of cadmium encountered in contaminated natural environments, slow cadmium uptake and binding to basal MT that is stabilized by cadmium and accumulates over time is a reasonable scenario for increases in MT concentrations observed in oysters in the Patuxent River. Whether induction is responsible for increases in MT accretion in other molluscan populations is open to question. For the purposes of using MT of this species as a 'biomarker', it appears based on this

analysis that emphasis should be placed on detection and quantification of the protein or its bound metals, rather than measurement of the mRNA.

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