Metallothionein turnover, cytosolic distribution and the uptake of Cd by the green mussel *Perna viridis*

T.Y.-T. Ng\textsuperscript{a}, P.S. Rainbow\textsuperscript{b}, C. Amiard-Triquet\textsuperscript{c}, J.C. Amiard\textsuperscript{c}, W.-X. Wang\textsuperscript{a,}\textsuperscript{∗}

\textsuperscript{a} Department of Biology, The Hong Kong University of Science and Technology (HKUST), Clear Water Bay, Kowloon, Hong Kong, China
\textsuperscript{b} Department of Zoology, The Natural History Museum, Cromwell Road, London SW7 5BD, United Kingdom
\textsuperscript{c} Université de Nantes, Nantes Atlantique Universités, Faculté de Pharmacie, SMAB, Service d’écotoxicologie, F-44000 Nantes, France

Received 30 October 2006; received in revised form 11 January 2007; accepted 27 January 2007

Abstract

We examined the relationship between Cd kinetics (uptake from solution and diet, and efflux), metallothionein turnover, and changes in the cytosolic distribution of accumulated Cd between protein fractions in the green mussel *Perna viridis*. We pre-exposed the mussels to 5, 20, 50 and 200\textsuperscript{μ}g g\textsuperscript{−1} of Cd for 1 week and determined the biokinetics of Cd uptake and efflux in the mussels. The dietary assimilation efficiency of Cd increased by 2 times following exposure to 20–200\textsuperscript{μ}g g\textsuperscript{−1} Cd, but the dissolved uptake rate was unchanged by pre-exposure to any Cd concentrations. The efflux rate of Cd was also similar among control and Cd pre-exposed mussels. The cytosolic distribution of Cd in the mussels that had been exposed to dissolved Cd, showed that besides metallothionein (7000–20,000 Da), high molecular weight proteins (>20,000 Da) were important for Cd binding and depuration. In general, the Cd pre-exposed mussels had higher metallothionein turnover with a higher metallothionein synthesis rate, but similar metallothionein breakdown rates as the control mussels. Metallothionein synthesis rate was correlated to the dietary assimilation of Cd, whereas metallothionein breakdown and Cd efflux rate were independent of each other. This study provides important new information for the role of metallothionein turnover on Cd kinetics in an aquatic invertebrate.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Cd; Kinetics; Cytosolic distribution; Metallothionein turnover; *Perna viridis*

1. Introduction

In marine bivalves cytosolic proteins play a vital role in metal sequestration, toxicity and elimination, albeit with variation among different species (Amiard et al., 2006). Metallothioneins (MTs) are cysteine-rich, metal-binding, heat-stable low molecular weight cytosolic proteins which play a role in the detoxification of certain trace metals including Ag, Cd, Cu, Hg and Zn. Cd and Cu in the cytosol of the clam *Ruditapes decussatus* had the greatest degree of binding to MT (Bebianno and Serafim, 2003). Furthermore, MT concentrations increased in the clam *Ruditapes philippinarum* and the green mussel *Perna viridis* after they were exposed to increasing concentrations of Ag (Ng and Wang, 2004) and Cd (Shi and Wang, 2005) respectively in the laboratory. In contrast, metals bound to non-MT cytosolic proteins such as high molecular weight (HMW) and low molecular weight (LMW) proteins have often been associated with deleterious effects at different biological levels, e.g., lipid peroxidation, growth reduction, reproduction impairment, behaviour modification and mortality (Couillard et al., 1995; Baudrimont et al., 1999; Wallace et al., 2000). In many previous studies, the role of MT has been emphasized, while the roles of other cytosolic proteins are often neglected. Our recent study provided preliminary evidence that heat-sensitive or HMW proteins can act as a temporary pool for storing metals when green mussels first encounter the metals (Ng and Wang, 2005a). Thus, it is of interest to examine any relationship between differences in uptake rate of a trace metal such as Cd after metal pre-exposure and associated differences in the distribution of the metal between cytosolic proteins.

MT is not only involved in metal detoxification processes (Amiard et al., 2006), but also plays an essential role in other metabolic processes (Mason and Jenkins, 1995). MT, like other proteins, will be degraded in lysosomes. The activation of the physiological process involving MT in response to a metal challenge might be reflected in increased turnover (synthesis and breakdown) of the protein, but not necessarily in changes in MT concentration (Mouneyrac et al., 2002). Such faster turnover...
may represent a dynamic steady state with proteins undergoing continuous and often selective breakdown. Metals released during the protein breakdown have the potential to bind to newly synthesized MT, so extending the turnover time of the metal in relation to the protein (Roesijadi, 1995; Mouneyrac et al., 2002). Thus in the oyster Crassostrea virginica, the biological half life of Cd bound to MT was 70 days, whereas the half life of the MT protein itself was only 4 or 20 days depending whether the estimate was made during or immediately after Cd exposure (Roesijadi and Klers, 1989; Roesijadi, 1995). As a result of the different affinities of the relevant metals for MT and the differential susceptibilities of MT-bound metals to lysosomal degradation, the uptake and excretion of metals may be affected to different degrees by changes in MT turnover rate. MT turnover has been investigated in the marine mussel Mytilus edulis (Bebianno and Langston, 1993) and the gastropod Littorina littorea (Bebianno and Langston, 1998). These earlier studies emphasized the relationship between MT breakdown and metal elimination, but no study has examined the relationship between MT synthesis and metal uptake kinetics.

In this study, we have attempted to investigate the relationships between MT turnover, changes in the cytosolic distribution of accumulated Cd and Cd kinetics (uptake from solution and diet, and efflux) in the green mussel P. viridis. Metal exposure was found to alter the Cd uptake kinetics by the mussels (Wang and Rainbow, 2005). In order to have a variation of uptake kinetics and MT concentration in the mussels, we pre-exposed the mussels to dissolved Cd for 1 week and determined the subsequent kinetics of Cd uptake using gamma-emitting 109Cd as a radiotracer. MT synthesis and breakdown were determined using beta-emitting L-[35S] cysteine as a marker since MT is atypically rich in the sulphur-bearing amino acid cysteine (Amiard et al., 2006) and 35S has been previously applied to determine MT breakdown in molluscs (Bebianno and Langston, 1993, 1998). Cd distribution among cytosolic proteins in the mussels was analyzed using high performance liquid chromatography.

2. Materials and methods

2.1. Collection of mussels and Cd pre-exposure

Green mussels P. viridis (shell length: 2.5–3 cm) were collected from Ma Liu Shui, Tolo Harbour, Hong Kong. The mussels were allowed to acclimate in aerated seawater (24°C, 33 psu) in the laboratory and fed with the diatom Thalassiosira pseudonana at a ration of 2% mussel tissue dry weight per day. After acclimation for 1 week, 50 mussels were transferred to 10l seawater without added Cd (control) or with stable Cd (5, 20, 50, or 200 μg l⁻¹) for 1 week. The series of Cd concentrations was selected for a variation of responses in the mussels. Although some concentrations (e.g., 50 and 200 μg l⁻¹) that we used in this study were 50–100× higher than environmentally realistic concentrations, we used these as an extreme scenario to investigate how mussels handled or detoxified Cd as the ambient Cd concentration increased. The different concentrations of Cd were prepared from CdCl₂·2H₂O (analytical grade, 1000 mg l⁻¹). Mussels were exposed to dissolved Cd for 16 h each day, then placed in clean seawater and fed with T. pseudonana for 8 h.

2.2. Concentrations of Cd and metallothionein (MT) in the mussels

Five mussels were taken from each treatment for the analysis of the accumulated Cd concentrations in the soft tissues after the week’s exposure. Mussels were rinsed with filtered seawater to remove the surface adsorbed Cd, then the soft tissue was dissected and dried at 80°C. Concentrated nitric acid (trace-metal grade) was added to digest the tissue at 110°C for 1 day. Then, the digest was diluted by 2% nitric acid and the Cd concentration in the samples was measured by inductively coupled plasma-mass spectrometry. Oyster tissue (standard reference material 1566b, National Institute of Standards and Technology) was similarly digested and used as a quality control of the metal analysis. Agreement was within 10%. The Cd concentration of mussels is expressed as μg g⁻¹ dry wt.

A silver saturation method (Scheuhammer and Cherian, 1991) is well established for measuring MT concentration in bivalves and was used to measure the MT (if any) induced by Cd in the mussel tissue. Five mussels from each treatment were dissected and the soft tissue weighed. The tissue was then homogenized in 3 ml Tris–base buffer (20 mM, pH 7) with 10 mM β-mercaptoethanol (antioxidant) and 0.1 mM phenyl-methylsulfonyl fluoride (protease inhibitor). The homogenate was centrifuged at 20,000 x g for 20 min at 4°C and separated into soluble (supernatant) and insoluble (pellet) fractions. The soluble fraction was used for the MT assay. Briefly, 0.5 ml 20 mg l⁻¹ stable Ag with 20 kBq ml⁻¹ 110mAg in 0.5 M glycine buffer was added to 0.3 ml of the homogenate soluble fraction to saturate the MT-binding sites for 10 min before analysis. Then excess Ag was removed by adding 0.1 ml rabbit blood cell haemolysate prepared as in Scheuhammer and Cherian (1986), followed by heat treatment at 100°C for 5 min and centrifugation at 785 x g for 5 min. The addition of haemolysate and heat treatment was repeated 2 times. Then, the supernatant was analyzed for 110mAg. MT concentration (μg g⁻¹ wet wt.) was calculated as 3.55 x the Ag concentration as in the mammalian tissue. MT recovery was >70%, using standard MT from rabbit liver (Sigma). The MT concentration was expressed as μg g⁻¹ wet wt. of tissue.

2.3. Assimilation of Cd from phytoplankton by the pre-exposed mussels

The dietary uptake of Cd from the phytoplankton was determined using our established methods for the mussels (Blackmore and Wang, 2002). The diatoms T. pseudonana were collected during the exponential phase of growth and added to 250 ml filtered seawater with f/2 nutrient (Guillard and Ryther, 1962) that did not contain EDTA, Cu or Zn. 109Cd (74 kBq l⁻¹, equivalent to 0.6 μg l⁻¹) was spiked into the medium and the diatoms were cultured for 4 days in order to attain uniform labelling of the cells. Cd concentration in the radiolabelled diatoms was about 1.2 μg g⁻¹. Cells were then filtered by a 3 μm
polycarbonate membrane and resuspended in filtered seawater twice to remove the surface loosely adsorbed Cd on the cells. Five mussels from each treatment were put individually into containers with 500 ml filtered seawater and acclimated for 5 min before feeding. Radiolabelled diatoms were added at 10 min intervals for 30 min. After feeding, the mussels were rinsed with filtered seawater and taken for \(^{109}\)Cd live counting. These mussels were then left for depuration in a 20 l seawater circulating tank. The depuration of \(^{109}\)Cd was monitored at 3, 7, 12, 24, 36 and 48 h. Faeces from the mussels were removed and unlabelled *T. pseudonana* was supplied to the mussels regularly. Assimilation efficiency (%, AE) was defined as the percentage of \(^{109}\)Cd retained in the mussels at 48 h.

### 2.4. Dissolved uptake of \(^{109}\)Cd and subcellular distribution of \(^{109}\)Cd and \(^{35}\)S

Three pre-exposed mussels from each treatment were placed into each of three replicate beakers with 400 ml filtered seawater spiked with 280 kBq \(^{109}\)Cd l\(^{-1}\) (equivalent to 2.22 \(\mu\)g l\(^{-1}\) Cd) and 460 kBq \(^{35}\)S l\(^{-1}\) (in the form of L-[\(^{35}\)S] cysteine, equivalent to 0.12 \(\mu\)M). Water was also dual labelled with L-[\(^{35}\)S] cysteine for uptake because we were interested in examining the distribution of L-[\(^{35}\)S] cysteine in the cytosolic proteins, especially in the MT-like protein (MTLP). One mussel and 4 ml water were sampled from each beaker at 0 (water only), 1, 2 and 3 h (water and mussels) for \(^{109}\)Cd and \(^{35}\)S analysis. The soft tissues of the mussels were dissected out, weighed wet and homogenized in 3 ml 20 mM Tris–base buffer (20 mM, pH 7, 150 mM NaCl) at a flow rate of 0.4 ml min\(^{-1}\). Although most studies monitor MT at a wavelength of 254 nm, we used 280 nm to detect the total protein because we were also interested in examining proteins other than MT, e.g., high molecular weight protein. When a peak appeared, the fraction was collected for \(^{109}\)Cd counting, followed by an addition of 3 ml scintillation cocktail (Wallac OptiPhase ‘HiSafe’ 3). The mixture was incubated in the dark overnight before analysis for \(^{35}\)S. We defined the cytosolic proteins according to their molecular size—HMW, MTLP and LMW proteins. Since the molecular size of MTLP is reported to range from 7000 to 20,000 Da in bivalves (Geret and Cosson, 2002; Ciocan and Rotchell, 2004), we defined the fractions with molecular sizes 7000–20,000 Da as MTLP, <7000 Da as LMW, >20,000 Da as HMW. Since MTLP is often separated from the heat-treated cytosol in most studies, we compared the chromatograms of the heat-treated cytosol and untreated cytosol of the mussels in our trial experiments. We found no difference between the two protein profiles, which may support that the protein separated with molecular size 7000–20,000 Da was only MTLP and thereafter, we did not heat-treat the cytosol for size exclusion chromatography. Counts of \(^{109}\)Cd or \(^{35}\)S in the tissue and cytosolic fractions were normalized to wet wt. of the tissue (cpm g\(^{-1}\)) and convert to concentrations (pg g\(^{-1}\)) for Cd and L-[\(^{35}\)S] cysteine.

### 2.5. Depuration of dissolved \(^{109}\)Cd and subcellular distribution of \(^{109}\)Cd and \(^{35}\)S

About 20 mussels from each Cd pre-exposure treatment were exposed to dissolved \(^{109}\)Cd and \(^{35}\)S with an additional spike of 0.46 kBq \(^{35}\)S ml\(^{-1}\) to ensure a high concentration of \(^{35}\)S in the water during the exposure period. Water was dual labelled with \(^{35}\)S for the similar reason as uptake. Water samples were taken for \(^{109}\)Cd and \(^{35}\)S analysis at the beginning and end of exposure. After 15 h, the water was replaced and the mussels were left for depuration in the clean seawater. Three mussels were immediately sampled for \(^{109}\)Cd and \(^{35}\)S analysis, with further samples being taken on days 1, 2, 3, 4 and 6 of depuration. Seawater was renewed daily. Mussels were dissected and homogenized as described above for \(^{109}\)Cd and \(^{35}\)S analysis in the cytosol, cytosolic proteins and insoluble fraction.

### 2.6. Radioactivity measurement and statistical analysis

\(^{109}\)Cd radioactivity was measured on a Wallac 1480 Wizard 3 in. Perkin-Elmer gamma counter at energy level 88 keV, whereas the radioactivity of \(^{35}\)S was measured on a Wallac WinSpectral 1414 Liquid Scintillation counter (Perkin-Elmer). Radioactivity was corrected for background, quenching effects and decay, and the samples were counted for 2–5 min. Since the samples were dual labelled with \(^{109}\)Cd and \(^{35}\)S, we tested the interference between gamma and beta emissions before the experiments. Results showed that \(^{35}\)S increased the \(^{109}\)Cd gamma activity by <0.1% and \(^{109}\)Cd increased \(^{35}\)S beta activity by <10%. Therefore, we ignored the interaction and used the counts directly without any correction.

Data were checked for homogeneity and normal distribution and the percentage data were arcsine transformed for parametric statistical analysis. One-way ANOVA was used to test for difference among groups of pre-exposed mussels \((P<0.05)\). Then, post hoc tests were applied to identify the differences between groups \((P<0.05)\). Regression analysis was used to determine the regression coefficient \((r^2)\) and significance \((P<0.05)\) of
relationships describing $^{109}\text{Cd}$ uptake rate in soft tissue, $^{109}\text{Cd}$ and L-[$^{35}\text{S}$] cysteine uptake or efflux rate in cytosolic proteins.

3. Results

3.1. Concentrations of Cd and MT in the pre-exposed mussels

Cd exposure increased the Cd concentration in the mussel soft tissues after 1 week. Cd exposure at or above 20 $\mu$g l$^{-1}$ for 1 week increased the tissue concentration significantly by 10–100 times (Table 1, $P<0.05$). MT concentration in the tissue showed an increasing trend as the Cd exposure concentration increased, but there was no statistically significant difference between the control and other treatments (Table 1, $P>0.05$) except that the MT concentration in the 50 $\mu$g l$^{-1}$ exposed mussels was significantly higher than in the 5 $\mu$g l$^{-1}$ exposed ones. When MT and Cd concentration was compared in the same treatment, MT concentrations were similar at Cd tissue levels <10 $\mu$g g$^{-1}$ dry wt., then increased rapidly and reached a plateau with Cd concentration in the tissue increasing from 20 to 200 $\mu$g Cd g$^{-1}$ dry wt.

3.2. Assimilation, dissolved uptake of Cd and subcellular distribution of $^{109}\text{Cd}$ and $^{35}\text{S}$

Dietary uptake of Cd in the mussels was affected by the pre-exposure. The AE of Cd was elevated by 2 times after 1 week’s pre-exposure to 20, 50 and 200 $\mu$g Cd l$^{-1}$ (Table 1, $P<0.01$).

The $^{109}\text{Cd}$ and $^{35}\text{S}$ counts in the water samples were similar over the period of dissolved uptake, indicating that there was constant supply of both Cd and cysteine for the mussels. Dissolved uptake of $^{109}\text{Cd}$ in the whole soft tissues was significantly correlated to the length of exposure; thus it was possible to calculate the uptake rate (slope constant of the linear regression against time during efflux). Standard error was expressed for Cd uptake rate and $k_e$ (slope ± standard error). Asterisk(s) indicate significant fits to linear regression lines with significance at $P<0.05$ (*) and $P<0.01$ (**). Different letters indicate significant difference between groups. Ctl: control and numbers after Cd are the pre-exposed concentrations at $\mu$g l$^{-1}$.

$^{109}\text{Cd}$ did not bind only to MTLP but was also present in the HMW fraction and to a lesser degree in the LMW fraction (Fig. 1). $^{109}\text{Cd}$ uptake increased linearly over time in the HMW protein of most mussels but few significant linear relationships were observed for $^{109}\text{Cd}$ uptake in the LMW and MTLP protein fractions. Taking into account the relationship between $^{109}\text{Cd}$ activity in each fraction and the length of exposure, the uptake rate in each fraction for each treatment was calculated from the significant linear regression curves (Table 2). Uptake rate of $^{109}\text{Cd}$ into the HMW protein was the highest among all the cytosolic proteins and furthermore, increase of uptake into cytosolic proteins was more apparent in the mussels pre-exposed to Cd. There were positive correlations between total $^{109}\text{Cd}$ in the cytosol and $^{109}\text{Cd}$ associated with individual cytosolic fractions, but the regression for $^{109}\text{Cd}$ associated with LMW was not strong (Fig. 2). Thus, MTLP and HMW proteins were both important for binding Cd when there was an increase of Cd taken up into the mussels.

L-[$^{35}\text{S}$] cysteine kinetics were used as a marker of MT turnover in the mussels. The majority of L-[$^{35}\text{S}$] cysteine was incorporated into MTLP and HMW protein (>85%), whereas very little was associated with LMW protein (5%). There was a higher distribution of L-[$^{35}\text{S}$] cysteine in the MTLP of the mussels pre-exposed to the higher concentrations of Cd (60% in MTLP of mussels exposed to 200 $\mu$g l$^{-1}$ Cd) (Fig. 1). L-[$^{35}\text{S}$] cysteine concentration increased only significantly and linearly with time in the MTLP of the 20 and 50 $\mu$g l$^{-1}$ Cd pre-exposed mussels, and the HMW protein of the 200 $\mu$g l$^{-1}$ Cd pre-exposed mussels. The uptake rate of L-[$^{35}\text{S}$] cysteine was similarly calculated as explained above for $^{109}\text{Cd}$ (Table 2), and was the highest in the MTLP and HMW fractions, but it was undetectable in the mussels pre-exposed to low Cd concentrations, e.g., Ctl-LMW and Ctl, Cd5-HMW fractions (Table 2). The increase of L-[$^{35}\text{S}$] cysteine also appeared more apparent in the mussels pre-exposed to the higher Cd concentrations.

3.3. Cd depuration and subcellular distribution of $^{109}\text{Cd}$ and $^{35}\text{S}$

Again, $^{109}\text{Cd}$ and L-[$^{35}\text{S}$] cysteine concentrations in the water were similar at the beginning and end of the 15 h exposure. During the 7 days of depuration, Cd pre-exposed mussels had similar

Table 1
Concentrations of Cd and metallothionein (MT) in the tissue (µg g$^{-1}$) and parameters of Cd kinetics in the mussel Perna viridis pre-exposed to dissolved Cd

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cd concentration (µg g$^{-1}$ dry wt.)</th>
<th>MT concentration (µg g$^{-1}$ wet wt.)</th>
<th>$^{109}\text{Cd}$ uptake rate from water (ng g$^{-1}$ h$^{-1}$)</th>
<th>AE (%)</th>
<th>$k_e$ (day$^{-1}$)</th>
</tr>
</thead>
</table>
Fig. 1. Concentrations of $^{109}$Cd and $l$-$[35S]$ cysteine (pg g$^{-1}$ wet wt.) bound to cytosolic proteins in *Perna viridis* over the uptake period. Mean ± standard deviation ($n = 3$). Regression lines were plotted when the regressions were significant ($P < 0.05$). Low molecular weight (LMW): <7000 Da; metallothionein-like protein (MTLP): 7000–20,000 Da; high molecular weight (HMW): >20,000 Da.Ctl: control and numbers after Cd are the pre-exposed concentrations at /H9262/gl$^{-1}$.

Table 2

Uptake or efflux rates of $^{109}$Cd and $l$-$[35S]$ cysteine (pg g$^{-1}$ h$^{-1}$) in cytosolic proteins of the mussel *P. viridis* pre-exposed to dissolved Cd

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$^{109}$Cd uptake rate (pg g$^{-1}$ h$^{-1}$)</th>
<th>$l$-$[35S]$ cysteine uptake rate (pg g$^{-1}$ h$^{-1}$)</th>
<th>$l$-$[35S]$ cysteine efflux rate (day$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMW</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctl</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cd5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cd20</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cd50</td>
<td>1.5 ± 0.5*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cd200</td>
<td>6.7 ± 0.1**</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MTLP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctl</td>
<td>ND</td>
<td>ND</td>
<td>$-1.3 ± 0.5*$</td>
</tr>
<tr>
<td>Cd5</td>
<td>3.5 ± 1.0*</td>
<td>ND</td>
<td>$-1.0 ± 0.3**$</td>
</tr>
<tr>
<td>Cd20</td>
<td>ND</td>
<td>32.5 ± 2.5**</td>
<td>$-1.1 ± 0.4**$</td>
</tr>
<tr>
<td>Cd50</td>
<td>ND</td>
<td>48.9 ± 6.3**</td>
<td>$-1.4 ± 0.4**$</td>
</tr>
<tr>
<td>Cd200</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HMW</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctl</td>
<td>4.0 ± 0.8*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cd5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cd20</td>
<td>5.0 ± 0.3*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cd50</td>
<td>6.6 ± 1.8*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cd200</td>
<td>4.7 ± 0.1*</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^{109}$Cd or $l$-$[35S]$ cysteine uptake or efflux rate was the slope of regression of $^{109}$Cd or $l$-$[35S]$ cysteine concentrations in each cytosolic protein against time. Standard error was expressed for $^{109}$Cd, $l$-$[35S]$ cysteine uptake and efflux rates (slope ± standard error). Asterisk(s) indicate significant fit to regression lines at $P < 0.05$ (*) and $P < 0.01$ (**). Low molecular weight (LMW): <7000 Da; metallothionein-like protein (MTLP): 7000–20,000 Da; high molecular weight (HMW): >20,000 Da.Ctl: control and numbers after Cd are the pre-exposed concentrations at /H9262/gl$^{-1}$. ND: not determined due to non-significant regressions or fluctuations in $l$-$[35S]$ cysteine concentration over time, wherever applicable.
efflux kinetics to each other and to the controls (Table 1). Cd was depurated at 3–6% each day. After 2 days of depuration, a relative Cd-enrichment in the cytosol (10–15% increase) was found in the control and Cd pre-exposed mussels ($P < 0.05$). This percentage then remained constant afterwards. $^{109}$Cd was depurated relatively similarly from the different protein fractions among the mussels, regardless of their pre-exposure history (Fig. 3). The concentration of $^{109}$Cd in the LMW proteins did not change significantly whereas the concentration in the MTLP and HMW proteins fluctuated over time. There was an obvious increase of $^{109}$Cd in the HMW proteins between day 0 and day 2, and an increase of $^{109}$Cd in MTLP between day 2 and day 4, especially in the mussels pre-exposed to higher Cd concentrations (50 and 200 $\mu$g l$^{-1}$). Since there was no consistent trend of $^{109}$Cd in the different protein fractions over time, we did not fit the data to any regression lines. When there was a reduction of $^{109}$Cd in the different protein fractions over time, we did not fit the data to any regression lines. When there was a reduction of $^{109}$Cd in the cytosol also reduced, but the relationship was weak for MTLP (Fig. 2).

Release of L-[35S] cysteine accompanied the depuration of Cd from the mussels (Fig. 3). L-[35S] cysteine associated with MTLP decreased exponentially. Only about 15% L-[35S] cysteine remained in the MTLP after 1 day, subsequently it became constant. The loss or efflux rate of L-[35S] cysteine from MTLP in general was independent of the exposure history, except for a non-significant regression relationship for mussels pre-exposed to 200 $\mu$g l$^{-1}$ Cd (Table 2, Fig. 3). The half life of MT was determined by dividing ln 2 over L-[35S] cysteine efflux rate and it ranged between 12 and 16 h for control and mussels pre-exposed to 5, 20 and 50 $\mu$g l$^{-1}$ Cd. About 50% of L-[35S] cysteine was lost from the LMW and HMW protein fractions over the depuration period. No regression lines were used to fit the data of LMW and HMW but a trend of decreasing L-[35S] cysteine was observed.

3.4. Correlation analysis

MT concentration in the mussels was positively related to L-[35S] cysteine uptake rate into MTLP (Fig. 4). L-[35S] cysteine uptake rate was also positively correlated to the Cd AE of the mussels (Fig. 4). However, L-[35S] cysteine synthesis rate was not related to the Cd dissolved uptake rate in the mussels.

4. Discussion

The accumulation rates of Cd and the effects of Cd pre-exposure on the green mussels were similar to those reported previously (Blackmore and Wang, 2002; Shi and Wang, 2005). The relationship of MT concentration and Cd soft tissue concentration of the mussels implies that MT is important for detoxifying Cd in green mussels, but the detoxification process was overwhelmed when the Cd exposure continued to increase (Mason and Jenkins, 1995). The mussels assimilated more Cd from ingested phytoplankton after the acute Cd pre-exposure, but the pre-exposure did not affect the Cd uptake kinetics from the water. Nevertheless, dissolved Cd was incorporated at different rates into the cytosolic proteins of mussels differentially pre-exposed to Cd.

Cytosol plays an important role in the binding of accumulated Cd in the mussels since about half of the labelled Cd was distributed among cytosolic proteins in the dissolved exposure, of which HMW proteins and MTLP were the dominant subcellular pools for Cd. Cd was distributed to the greatest extent to HMW protein, and uptake of Cd into this pool showed the most significant relationships with time. The same pattern of Cd penetrating HMW proteins at the beginning of the exposure was shown in different bivalves (Evtushenko et al., 1986 and literature cited therein). In this study, we used 10 mM mercaptoethanol as reducing agent that protected the structure of MT and prevented the aggregation of proteins during protein extraction. It has been suggested that the presence of reducing agents may be responsible for increased metal binding in the LMW proteins of the bivalves (Roesijadi and Drum, 1982; Lobel, 1989; Bragigand and Berthet, 2003), thus causing artifacts on the separation of metal-binding proteins. On the other hand, most authors consider
Fig. 3. $^{109}$Cd and $\text{L-}[\text{S}^{35}]$ cysteine concentrations (pg g$^{-1}$ wet wt.) in the cytosolic proteins of P. viridis during depuration. Mean $\pm$ standard deviation ($n=3$). Regression lines were plotted when the regressions were significant ($P<0.05$). No regression was fitted for $^{109}$Cd and $\text{L-}[\text{S}^{35}]$ cysteine concentrations in LMW and HMW proteins due to fluctuation trends over time. Low molecular weight (LMW): $<7000$ Da; metallothionein-like protein (MTLP): $7000–20,000$ Da; high molecular weight (HMW): $>20,000$ Da. Ctl: control and numbers after Cd are the pre-exposed concentrations at $/H9262\text{g}^{-1}$.

Fig. 4. Correlations between uptake rate of $\text{L-}[\text{S}^{35}]$ cysteine (pg g$^{-1}$ h$^{-1}$) in metallothionein-like protein (MTLP) or MT concentration (µg g$^{-1}$ wet wt.) and Cd assimilation efficiency (% AE) in P. viridis. Regression lines were plotted when the regressions were significant ($P<0.05$). Note that $\text{L-}[\text{S}^{35}]$ cysteine uptake rate was calculated for correlation analysis in the control and mussels pre-exposed to 5 and 200 µg l$^{-1}$ Cd although the regressions of $\text{L-}[\text{S}^{35}]$ cysteine uptake against time (Fig. 1) were not significant. Asterisk indicates significant regressions at $P<0.05$ ($^*$).

that mercaptoethanol plays a protective role for MT structure, allowing the preservation of metal binding to MT during chromatography (Minkel et al., 1980; Lobel, 1989; Carpene, 1993; Bragigand and Berthet, 2003). In the present work, any potential artifactual effect of mercaptoethanol would have led to an overestimate of Cd binding in the LMW fraction, a bias that would not have promoted the observed comparatively higher importance of MT and HMW proteins in Cd binding. Cd pre-exposed mussels had more significant uptake of Cd into cytosolic proteins over time, compared to the control mussels, implying that the mussels were more efficient in handling the metals following metal pre-exposure. In addition, the significant correlations between total cytosol Cd concentrations and Cd concentrations bound to MTLP during uptake agreed with the previous studies on Cd accumulation in aquatic invertebrates (Mason and Jenkins, 1995; Amiard et al., 2006).

Wallace et al. (2003) defined the organelles and heat-sensitive proteins (e.g., metalloenzymes) as metal sensitive subcellular metal-binding fractions, considering these to be the sensitive components of the cells. The non-specific binding of metals to non-MT fractions can imply the onset of cellular toxicity (e.g., HMW proteins). Such binding of metal to proteins other than detoxificatory ones (including MT) is often described as “spill over” (Winge et al., 1974; Couillard et al., 1995; Baudrimont...
et al., 1999). Spill over is often a feature apparent in some laboratory exposures when organisms are exposed to raised concentrations of metals. The slower uptake rate of Cd into the cytosolic proteins of the mussels exposed to 200 μg l⁻¹ Cd may be explained by a toxic effect of the acute exposure on metal subcellular distribution or detoxification processes. This can further be supported by the saturation of MT concentration at high Cd concentrations in the mussel soft tissues. Under the conditions of our experiments, Cd binding to HMW proteins occurred without MT-overloading, therefore Cd may bind to some HMW proteins specifically and similar data were reported for scallops (Evtushenko et al., 1986). Such proteins are supposed to be analogous to albumin and globulin of blood serum – where metal can be bound to an amino acid carboxyl group – and can participate in metal transport (Robinson and Ryan, 1988). Since green mussels distributed a substantial amount of Cd into HMW and the increase of Cd in the cytosol correlated positively with increase of Cd in HMW protein, HMW appears to be the chief protein fraction for binding Cd before toxic effects occur. Our previous study also found a higher percentage of Cd associated in the HMW proteins of green mussels with higher dissolved uptake rates (Ng and Wang, 2005a). Therefore, both studies reinforce the importance of HMW proteins for short-term metal binding during uptake in the mussels.

Increased excretion of Cd was apparently not a defensive response in the mussels since the efflux rates of Cd by the mussels pre-exposed to dissolved Cd were similar to that of the control. In general, the efflux rate measured in this study was slightly faster than previously reported in green mussels (0.007−0.029 day⁻¹) (Blackmore and Wang, 2002; Ng and Wang, 2005b), which may be explained by the shorter radiolabelling period used (15 h as opposed to days of radiolabelling before depuration). Previous studies have reported the importance of cytosol binding of metals on metal elimination. For example, Cd in the soluble fraction of the oysters was more easily eliminated than Cd in insoluble form (Roesijadi and Klerks, 1989; Geffard et al., 2002). Our results also found an increase of Cd in the cytosol of the green mussels during the depuration period. This was not due to biological variation of the mussels because the increase was observed in mussels from all treatments. Therefore Cd was probably shifted from the insoluble fraction to the cytosol as supported by the reduction and increase of Cd in the insoluble fraction and cytosol respectively in the first 2 days of depuration, but a relatively constant ¹⁰⁹Cd concentration in the cytosol. Among the cytosolic proteins, part of the Cd initially associated with HMW protein, shifted to MTLP during the later period of depuration (2–4 days), similar to our earlier studies on the dynamics of metals during depuration in green mussels (Ng and Wang, 2005a). Metals may first bind to HMW proteins, but move to the MTLP for detoxification or long-term storage. Baudrimont et al. (2003) and Bebianno et al. (1994) suggested that MT may be involved in metal elimination in bivalves, whereas our study only showed a temporary binding of Cd to MTLP. This may be explained by the MT turnover cycle that has been rarely considered in previous studies.

L⁻[³⁵S] cysteine is proved to be an excellent marker for MT turnover in the green mussels because there was a high percentage of L⁻[³⁵S] cysteine in MTLP and a significant incorporation of L⁻[³⁵S] cysteine into the MTLP of the Cd pre-exposed mussels over time. In addition, the twofold increase of L⁻[³⁵S] cysteine incorporation rate in MTLP of the Cd exposed mussels (e.g., 50 and 200 μg l⁻¹) compared to the control mussels corresponded to the almost same fold increase of MT concentration. Therefore, incorporation of L⁻[³⁵S] cysteine into MTLP does appear to be a good index of the MT synthesis rate of the green mussels. Based on our L⁻[³⁵S] cysteine data, the MT breakdown of the green mussels was extremely fast (12–16 h), compared to other aquatic invertebrates. Previous studies demonstrated that MT is degraded more quickly in mammals, e.g., 3–5 days in rats exposed to Cd (Chen et al., 1975) than in marine molluscs, e.g., 7 days in C. virginica (Roesijadi et al., 1991), 25 days in M. edulis (Bebianno and Langston, 1993) and 69–190 days in L. littorea (Bebianno and Langston, 1998). The short half life of MT that we obtained may be chiefly explained by the different methods or approaches used to determine MT breakdown compared to previous studies. Bebianno and Langston (1993, 1998) injected L⁻[³⁵S] cysteine into the tissue or mantle cavity of the mussels and snails, then waited a few days for equilibrium before examining the depuration of ³⁵S weekly for a month. Since their observation interval was longer, the MT measurement may be a net product of MT synthesis and breakdown within the week (i.e. a steady-state measurement). In this study, we used a kinetic approach to measure the instant breakdown of MT within 7 days. Generally, the half-life of MT is shorter than the half lives of associated metals. Metals bound to Mt will be transported to lysosomes where MT is degraded. Metals would then be released back to the cytosol and induce MT synthesis again (Viarengo and Nott, 1993). In this study, such a cycle of Cd binding and release from the MTLP of green mussels was also observed during depuration. ³⁵S in fact has been widely used to examine the synthesis and breakdown of proteins other than MT in many organisms (Hawkins et al., 1987; Barnes et al., 2002). We used ³⁵S in the form of cysteine in this study, in order to increase incorporation into MT, but significant incorporation of ³⁵S was still obtained in other proteins (e.g., HMW), a potential artifact of using ³⁵S as a marker for MT turnover in the bivalves. In addition, ¹⁰⁹Cd present in the insoluble fraction is at least partly sequestered in the lysosomal vacuolar system which is eliminated by exocytosis. However, the importance of this excretion pathway is probably less important for Cd than for other metals (Viarengo and Nott, 1993 and literature cited therein).

To conclude, both MTLP and HMW proteins are important for the binding and depuration of Cd in green mussels. The Cd pre-exposed mussels had a faster MT synthesis but a similar MT breakdown rate to the control mussels. Therefore, the resultant MT concentration was also higher in the Cd exposed mussels. The MT synthesis rate was correlated to the assimilation of Cd from phytoplankton. In addition, the Cd efflux rate was independent of the MT breakdown rate due to the much faster degradation of MT and the possible re-binding of Cd to new MT. Our study has significant implications for further investigations into MT turnover in aquatic invertebrates. A metal challenge may increase the rate of the MT turnover processes, e.g., synthesis
or breakdown, but the role of each process on metal kinetics has often been neglected in previous studies.

Acknowledgement

This study was supported by a Competitive Earmarked Research Grant from the Hong Kong Research Grants Council (HKUST6405/05M) to W.-X. Wang.

References


