BIOCHEMICAL ADAPTATION

Biochemical Mechanisms of Adaptation to Cadmium and Copper Ions in the Mussel *Mytilus trossulus*

V. P. Chelomin, N. N. Belcheva, and M. V. Zakhartsev

Pacific Institute of Oceanology, Far East Division, Russian Academy of Sciences,
Baltiyskaya ul. 43, Vladivostok, 690041 Russia

Received February 26, 1997

Abstract—General biochemical mechanisms for heavy metals adaptation are shown using the example of the marine mollusk *Mytilus trossulus*. Mussels were adapted to low concentrations of copper and cadmium under laboratory conditions. Cu and Cd preexposure led to enhanced tolerance to mercury toxicity (Hg bioassay) and alterations in the cellular biochemical apparatus of the gills and the hepatopancreas. In addition to metallothioneins, which are the central component of induced tolerance, the glutathione system is involved in all proposed schemes of metal detoxification. A comparison of the kinetics of metal accumulation by the tissues of adapted and unadapted mussels allows us to suggest that the adaptation mechanism uses metal transport from the gills to the hepatopancreas.

A large array of chemical substances contaminate the aquatic environment, but heavy metals and their compounds are a real threat to aquatic organisms. The hazard is due not only to their biological activity, but also their accumulation in various components of the ecosystems. In contrast to organic contaminants, which in the course of time are utilized and eliminated from the biosphere, heavy metals are capable of preserving their biological activity virtually infinitely. Therefore, the problem of elucidation and study of physiological and biochemical systems ensuring the adaptability and tolerance of aquatic organisms to increasing environmental levels of heavy metals remains, in essence, a problem of general biology, but under conditions of increased anthropogenic impacts on the biosphere, it has acquired a particular practical importance.

Among a variety of factors that determine the character of interactions of the organism with a chemical compound, the most important are the peculiarities of metabolism of a given biological system, which are manifested in specific adaptive detoxification biochemical responses. In biological systems, the effectiveness of these reactions determines the diversity of forms of reactivity to various toxicants, from highly sensitive to resistant.

To define the degree of sensitivity, the term "tolerance" is generally adopted. In recent experimental aquatic toxicology, it refers to phenotypic plasticity of survival of individuals exposed to lethal conditions [18, 35]. As applied to heavy metals, tolerance of the organism is assessed as the maximum concentration of a metal at which the organism survives for a specific time or as the time of survival at lethal concentration.

Differently advanced biological systems, from plants to mammals [3], including lower vertebrates [14], fish [20], terrestrial and aquatic invertebrates [34], are able to enhance their tolerance to heavy metals after preexposure (acclimation) to low metal levels. This phenomenon is called "induced tolerance" in the literature. The potential capacity for induced tolerance has been reported in review works [18, 30, 35] summing up the results from laboratory studies on the acclimation of some fish and invertebrates to sublethal concentrations of Zn, Cu, and Cd. It has been suggested [13, 33] that induced tolerance can occur as well in organisms exposed to elevated environmental metal levels [13, 33].

To explain this, the majority of investigators concentrate their attention on the ability of organisms to synthesize specific, low molecular weight, cysteine-rich, metal-binding proteins [3, 14, 18], which are called metallothioneins (MTs).

The detoxification role of MTs is accounted for by their ability to form stable complexes with heavy metals, thus protecting intracellular structures and biochemical systems against lesions [5, 11]. In a number of cases, metal tolerance correlates with general synthesis of MTs or similar proteins, providing a convincing proof of their importance in the defense processes. Incidentally, there are a few reports indicating the involvement of various biochemical systems in the formation of tolerance [1, 17, 30]. Generalization of these fragmentary data suggests that metal detoxification involving MTs is a response in a complex system of interlinked biochemical responses that ensures the cell's heavy metal tolerance. To substantiate this opinion, we provide literature data and original experimental results from the study of adaptive biochemical responses to heavy metals in the marine bivalve *Mytilus trossulus*.
To this end, mussels were experimentally acclimated to low concentrations of copper and cadmium. After metal adaptation was elicited, we performed a series of experiments using the “load” approach to elucidate the primary mechanism of adaptation.

MATERIALS AND METHODS

Adult mussels *Mytilus trossulus* were collected in Peter the Great Bay around Popov Island (Sea of Japan), thoroughly selected by size (4.5–5.0 cm), and maintained in 140-l aquaria for at least 10 days prior to experiments.

For experimentation, mussels were divided into five groups, with 30 in each. The first two mussel groups were maintained for 15 days in 40-l aquaria with relatively low concentrations of metals: 5 µg Cu/l for group 1 and 25 µg Cd/l for group 2. Upon termination of this period, mussels were kept for 21 days in aquaria containing elevated metal concentrations: 50 µg Cu/l for group 1 and 250 µg Cd/l for group 2. Below, groups 1 and 2 are referred to as “adapted” or “acclimated” mussels. Another two groups were first maintained in normal unfiltered seawater for 15 days, and on day 21 they were transferred in aquaria with elevated metal concentrations: 50 µg Cu/l for group 3 and 250 µg Cd/l for group 4. We called them “unadapted” or “unacclimated” mussels. Group 5 of “intact” mussels was kept in an aquarium to which no metals were added. In all, four series of experiments were run.

The scheme of tolerance induction experiments was that proposed by Roesijadi and Fellingham [35] with some modifications. Since *M. trossulus* is highly sensitive to mercury, concentration of the metal was lowered from 75 to 50 µg/l in the acclimation bioassay.

Experiments were carried out at fairly constant water temperature (about 17–18°C) and constant metal levels; the water was changed periodically (after 48 h).

After definite periods of time (3, 7, 10, 14, 18, and 21 days), five specimens of each group were taken for determination of the content of metals (Cu and Cd), glutathione (GSH) and metallothioneins (MTs) in the gills and the hepatopancreas. To measure these biochemical indices, samples of each tissue (gills and hepatopancreas) of five mussels were pooled and averaged, and then determinations were made. All numerical data are mean values for four series of experiments ± standard deviation. The level of MT-proteins was determined using the Ellman reagent after precipitation of proteins by 7% trichloroacetic acid [27]. The concentrations of Cu and Cd in tissues were measured by atomic absorption spectroscopy after acid ashing of the sample [16]. The concentration of metals was estimated per dry tissue weight after drying to constant weight at 85°C.

Table 1. The content of glutathione (µg/mg protein) in mussel tissues after acclimation to copper and cadmium

<table>
<thead>
<tr>
<th>Group of mussels</th>
<th>Hepatopancreas</th>
<th>Gills</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>7.8 ± 0.06</td>
<td>2.10 ± 0.07</td>
</tr>
<tr>
<td>Cu-adapted</td>
<td>8.6 ± 0.09</td>
<td>3.15 ± 0.05</td>
</tr>
<tr>
<td>Cd-adapted</td>
<td>11.8 ± 0.07</td>
<td>2.70 ± 0.06</td>
</tr>
</tbody>
</table>

RESULTS

Tolerance induction was determined from the change in survival time at lethal mercury concentration (Hg bioassay). The results showed that the survival time in acclimated mussels was markedly longer than that of intact mussels. Half of the intact mussels perished within 7 days, while in Cu- and Cd-acclimated mussels, the survival time was 10 and 13 days, respectively.

Acclimation of mussels to relatively low levels of copper was accompanied by no significant accumulation of this metal: from 7.8 ± 2.6 to 10.5 ± 2.2 µg/g for gills and from 12.4 ± 1.9 to 15.8 ± 2.4 µg/g for hepatopancreas. At the same time, in the process of cadmium acclimation the concentration of this element in both organs sharply increased from 0.58 ± 0.17 to 51.3 ± 8.2 µg/g for the gills and from 1.65 ± 0.44 to 132.7 ± 14.3 µg/g for the hepatopancreas.

An analogous situation was observed when analyzing the changes in MT content in mussel tissues after adaptation to low metal concentrations. No significant changes in the MT content in the gill (from 0.54 ± 0.17 to 0.58 ± 0.11 µg/g) and the hepatopancreas (0.82 ± 0.21 to 0.86 ± 0.13 µg/g) were observed in the process of adaptation. During Cd acclimation, the amount of MTs in the gills changed slightly by the end of the experiment (from 0.54 ± 0.17 to 0.66 ± 0.10 µg/g), while in the hepatopancreas cells the MT content increased almost twofold (from 0.82 ± 0.21 to 1.76 ± 0.25 µg/g).

The content of glutathione in mussel is tissue-specific; the hepatopancreas cells contained four times as much glutathione as the gill cells (7.8 ± 0.06 and 2.1 ± 0.07 µg/mg protein, respectively). The level of glutathione increased to a varying degree, in the cells of both organs (Table 1), but Cd-induced tolerance was accompanied by a higher increase in the glutathione content in the hepatopancreas (1.5 times) than in the gills (1.3 times). At low level Cu exposures, the glutathione system of the gill cells was more responsive (about 1.5 times), and the hepatopancreas cells responded very slightly (1.1 times).

To elucidate the adaptive potential of mussels, we used the load method based on a comparison of the behavior of biochemical systems in adapted and unadapted mussels at high metal concentrations. The kinetics of accumulation of copper and cadmium by mussel tissue (especially during the first week of the
mussels, i.e., those transferred from normal seawater to aquaria with high metal concentrations, stored both elements in the gills 1.5 times more intensively than the hepatopancreas (Fig. 1). However, mussels which were preexposed to low concentrations of copper and cadmium exhibited the reverse tendency: both metals were accumulated to a greater degree in the hepatopancreas cells.

The character of changes in GSH in adapted and unadapted mussels in response to high metal levels differed depending on tissue and metal (Figs. 2, 3). During Cu exposure of unadapted mollusks, the amount of glutathione in the hepatopancreas cells gradually fell, reaching 48% of the initial value by the end of the experiment. A different situation was observed in the gill cells of the same mussels. During the first two weeks, the GSH level increased somewhat and then sharply fell to 60% of the initial amount. In adapted mussels, there was also a monotonous decrease in the glutathione level in the hepatopancreas cells, although at a much slower rate. In the gill cells of these mussels, the amount of GSH fell (by 20%) within the first days of experiment and later remained at a constant level (Fig. 2). In the Cd experiment, the most significant changes in the glutathione content occurred only in the hepatopancreas of acclimated and unacclimated mussels (Fig. 3). It is significant that in adapted mussels the amount of GSH in this tissue increased almost three times towards the end of the experiment and 1.7 times in unadapted mussels. In the gills, especially in adapted mussels, the level of glutathione fell (by 30%) during the first days of the experiment.

In contrast to the changes in the glutathione content, there were general tendencies in the synthesis of metallothionein proteins in the gill and hepatopancreas cells in adapted and unadapted mussels at high metal concentrations (Table 2). In Cu experiments, the MT content in the gills and the hepatopancreas increased two times by the end of the experiment for unadapted mussels and four times for adapted mussels. In the process of Cd accumulation, the content of these proteins in the gills and the hepatopancreas of unacclimated mussels increased by three and four times, respectively. In acclimated mussels, MT synthesis was stimulated more intensively, and by day 21 the amount of MT increased by four times in the gill cells and six times in the hepatopancreas cells, compared to the intact mussels.

DISCUSSION

Biochemical mechanisms of tolerance to heavy metals are nonspecific; i.e., they are general for both physiologically important metals (Cu and Zn) and typical toxic elements that do not participate in biological processes (Hg and Cd) [30, 35]. This is clearly illustrated by marine bivalves, particularly the mussel, which is a convenient subject for toxicity tolerance bioassays [13, 34, 35]. Acclimation of the mussels Mytilus edulis to low concentrations of Hg, Cu, Cd, and Zn...
Table 2. Content of metallothioneins (μg/mg protein) in tissues of adapted and unadapted mussels in conditions of accumulation of copper (50 μg/l) and cadmium (250 μg/l)

<table>
<thead>
<tr>
<th>Metal</th>
<th>Unadapted mussels</th>
<th>Adapted mussels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gills</td>
<td>hepatopancreas</td>
</tr>
<tr>
<td>Cu</td>
<td>1.10 ± 0.14</td>
<td>1.90 ± 0.20</td>
</tr>
<tr>
<td>Cd</td>
<td>1.90 ± 0.15</td>
<td>3.20 ± 0.19</td>
</tr>
</tbody>
</table>

markedly increased their survival time during exposure to lethal concentrations of mercury in water [34, 35]. Taking the methods of Roesijadi et al. as our basis, we found that following a short-term adaptation to sublethal levels of Cu and Cd Mytilus trossulus exhibited enhanced tolerance to mercury.

It is assumed that specific proteins, metallothioneins, are the central component of biochemical detoxification of metal ions. Many investigators believe that MTs are involved in the development of enhanced tolerance to heavy metals [3, 14, 18]. In a number of cases, enhanced metal tolerance correlates with general synthesis of MTs or related proteins [3, 10, 18, 20]. We also observed a similar tendency for the mussel Mytilus trossulus. This is particularly true for Cd experiments (Table 2). It should be emphasized that the MT amount increased, especially in the hepatopancreas, not only during preadaptation, but in response to high level Cd exposure as well; adapted mussels synthesized larger amounts of these proteins than unadapted mussels.

During acclimation to low Cd doses, MT presynthesis is stimulated [10]. This presynthesis may result in the production of excessive amounts of these proteins (the "overproduction" hypothesis) [19], ensuring the defense of organism transferred to a medium with high metal concentration. It is not inconceivable that metal binding is due not only to "de novo" synthesis of MT-like proteins, but also to competitive substitution of weakly bound metal from pre-synthesized molecules of MTs [10, 23]. This assumption is based on a comparison of the stability constants of various metal-MT complexes that constitute the following series: Ag > Hg > Cu > Cd > Zn [5]. Despite the peculiarities of the physicochemical properties of metal-binding proteins in marine invertebrates, bivalves in particular, this series holds true for them [32]. Hence, Cd-MT complexes formed during preadaptation are acceptors for mercury ions, which evidently leads to the decrease in toxicity observed in adapted mussels.

In Cu experiments, enhanced tolerance following acclimation was not accompanied by a significant change in the content of MT-like proteins. At the same time, when unacclimated and acclimated mussels were transferred to a medium with increased Cu concentration, acclimated mussels synthesized greater amounts of MTs (Table 2). This may indicate that at the preadaptation stage such processes as MT gene amplification [30] and MT mRNA synthesis [3] were activated. This preadaptation may contribute to a reduction in the induction time and increase the protective potential of the entire detoxification system under extreme conditions.

Recognizing the leading role of MT-like proteins in biochemical mechanisms of tolerance, we cannot ignore a few, but often controversial facts that, nevertheless, attest to a variety of adaptive processes aimed at enhancing tolerance to metal toxicity. These adaptations include changes in the transport properties of cell membranes, which are evident in decreased absorption [1, 18] or in the stimulation of mechanisms of metal excretion by the cells [18, 30]; the synthesis of metal-tolerant biomolecules, particularly enzymes; and the development of processes of storage of elements in granules of various origin [30]. There is convincing evidence for a correlation between the carotenoid content in tissue of Perna viridis and heavy metal tolerance of this mollusk [22].

Among biomolecules and biochemical systems involved in detoxification and, perhaps, metal tolerance, glutathione occupies a special place. Glutathione is the major nonprotein thiol in all biological systems due to its participation in the regulation of the intracellular reduction–oxidation equilibrium and relevant biochemical and physiological processes. GSH is involved in peroxide metabolism, the transport and detoxification of organic xenobiotics, and the regulation of protein synthesis [21, 26]. A close relationship between cell tolerance to various heavy metals and glutathione content has been established for warm-blooded animals [6, 9, 36]. Kang and Enger [17] suggested that the GSH level is the controlling factor in the modulation of cytotoxic response, especially at the initial stage of Cd exposure. This assumption is in agreement with the hypothesis of Singhal et al. [37], which was further elaborated by several research teams [8, 39]. This hypothesis holds that the glutathione system (glutathione and enzymes involved in its metabolism) is the cell's first major line of general defense against heavy metals, which is particularly important before the induction of metallothionein synthesis occurs. As with MTs, the detoxification mechanism uses the ability of glutathione and its oxidized form (glutathione disulfide, GSSG) to form chelate complexes with various metals, particularly Cu and Cd, which have high stability constants [28, 29]. The potential capacity of the glutathione system is great, taking into account the high concentration of glutathione in the cells (0.5 to 10 mM)
Since copper can be transferred from decreased content in the cell. In addition to the coordination and binding of copper, this reaction is also important later on, since copper can be transferred from the glutathione complex into the molecule of metallothionein, which can chelate the reduced form of copper [4, 8].

Comparison of the kinetics of metal accumulation by the tissues of adapted and unadapted mussels in the "load" experiment has revealed an interesting peculiarity. In preadapted mussels, the rate of metal accumulation by the gills decreased and, concurrently, there was a rise in this process in the hepatopancreas (Fig. 1). Metal storage in the tissues results from the unbalance between uptake and excretion. In mussels, as in most aquatic organisms, the gills are the main organ through which various heavy metals enter the organism via nutrients and are then distributed among the tissues [2, 7, 15]. Therefore, it can be assumed that preadaptation to relatively low metal concentrations not only leads to enhanced synthesis of intracellular specific metal-binding structures (MTs, GSH), but also facilitates the activation of the tissue transport system. The redistribution of increased element fluxes among tissues can be mediated by hemocyes found in mollusk hemolymph [25] and high-molecular-weight proteins that have a high cadmium-binding potential [31].

To illustrate this assumption, we provided data on the kinetics of accumulation of both metals in the form of the ratio of hepatopancreas/gill metal levels during 21 days of exposure of adapted and unadapted mussels to high metal concentrations (Fig. 4). These curves characterize the degree of concordance of the processes of accumulation and detoxification of the metals studied in the mussel tissues. In unadapted mussels, the disproportion in metal accumulation observed in acute experiment leads to a major load in the gill cells. In this case, metal influx from the surrounding medium to the organism through the gills is evidently not balanced by the level of the tissue transport system. In preacclimated mussels, metal accumulation proceeds without serious disturbances of the ratio of metal concentrations in tissues (Fig. 4). In other words, in acclimated mussels, an intensive flux of metals occurred from vulnerable tissue (gills) to tissues with a well-developed system of detoxification and storage (hepatopancreas and kidney). Such a redistribution of metals allows the organism to endure additional loads, thereby eventually providing enhanced tolerance.

It is not yet clear what biochemical mechanisms provide for the coordinated function of individual biological structures involved in metal detoxification, especially those such as GSH, MTs, metal transport proteins, and metal-storing membrane formations. However, it is evident that metal redistribution among the organs is a manifestation of a general mechanism of regulation inside the organism, in which intracellular structures and extracellular ligands augment each other.

**REFERENCES**


