

**Effect of cadmium and glutathione on malic enzyme activity in brown shrimps (*Crangon crangon*) from the Gulf of Gdańsk\***

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**Abstract**

The high level of cadmium in the abdominal muscle of the brown shrimp *Crangon crangon* is due to the serious pollution of the water in the Gulf of Gdańsk. The inhibition of malic enzyme (ME) activity by cadmium, and in consequence

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the reduced formation of NADPH, could interfere with cellular mechanisms for detoxifying the organism and reducing oxidative stress. The reduced glutathione (GSH) concentration in the abdominal muscle of *C. crangon* was calculated to be 5.8 mM. The objective of this study was to evaluate the part played by GSH in the effect of cadmium on the activity of NADP-dependent malic enzyme from abdominal muscles of brown shrimps. This enzyme is activated by certain divalent cations (Mg, Mn). The results demonstrate that cadmium inhibits ME activity from shrimp muscle, and that GSH and albumin can reduce this cadmium-inhibited NADP-dependent malic enzyme activity.

## 1. Introduction

Cadmium, a toxic heavy metal, adversely affects the condition and hence the reproduction of animals. Nevertheless, its effects on some cellular processes and its exact mode of action are still not fully understood (for a review, see Waisberg et al. (2003), Castro-Gonzales & Mendez-Armenta (2008)).

In shrimps kept at a cadmium concentration close to  $LC_{50}$  the activity of malic enzyme (ME) per gram wet weight of abdominal muscles was significantly higher than in the control group (Napierska et al. 1997). This short-term exposure also causes a concentration-dependent induction of metallothionein (MT) in shrimp abdominal muscles (Napierska & Radłowska 1998). MT is known to alter the toxicity of cadmium. In the muscles of crustaceans ME, which is activated by divalent cations, is involved in the formation of NADPH in the reversible decarboxylation of malate to form pyruvate in the presence of NADP (Skorkowski et al. 1980).

Glutathione (GSH), an important intracellular tripeptide (containing a thiol group with an affinity for heavy metals) present in cells (up to 8 mM), plays a key role in maintaining cellular homeostasis and protects the cell against xenobiotics, reactive electrophiles and oxidative stress (Viarengo et al. 1991, Griffith 1999, Dickinson et al. 2002, Kala et al. 2004, Habib et al. 2007). It was shown earlier that the GSH content decreased in the tissues of aging marine mussels. The age-related decrease in the level of this thiol-rich tripeptide is largely due to the reduction in the rate of its synthesis (Canesi & Viarengo 1997). Lange et al. (2002) showed that the hepatic level of total GSH increased in rainbow trout after 14 days' exposure to Cd by about 1.5 times compared to the control, but after 28 days no significant changes were observed. Gil & Pla (2001) postulated that GSH could serve as a biomarker for a variety of xenobiotics.

In order to gain a better understanding of the part played by GSH in protecting malic enzyme from cadmium toxicity, we studied how the GSH level would affect the inhibition of malic enzyme activity by cadmium. In the muscles of crustaceans this enzyme is involved in NADPH formation,

which is important in detoxification processes. The toxic effect of cadmium was tested in vitro by using the NADP-dependent malic enzyme, activated by divalent cations, from shrimp abdominal muscles. Some of our results suggest that the presence of cellular GSH reduces cadmium inhibition of NADP-dependent malic enzyme and in consequence protects this enzyme.

## 2. Material and methods

Brown shrimps *Crangon crangon* 3–4 cm in length were caught in the Gulf of Gdańsk off Sobieszewo Island near the delta of the River Vistula in June and July and kept in aerated seawater.

### 2.1. Enzyme and protein assays

Malic enzyme (L-malate: NADP oxidoreductase (decarboxylating) E.C. 1.1.1.40) activity at all purification steps was tracked spectrophotometrically with a UV-VIS recording spectrophotometer by observing the appearance of NADPH at 340 nm and 25°C. The standard reaction mixture contained 50 mM Tris-HCl, pH 7.5, 0.5 mM NADP, 5 mM L-malate and 1 mM manganese chloride. Enzyme activities were calculated using  $E \text{ mM} \times 340^{-1} = 6.22$  for NADPH in a 1 cm light-path quartz cell. Protein concentration was determined by Spector's (1978) method.

### 2.2. NADP-dependent malic enzyme purification

Shrimp malic enzyme (ME) (L-Malate: NADP oxidoreductase (decarboxylating) EC 1.1.1.40) was isolated from the abdominal muscles of brown shrimps *C. crangon* caught in the Gulf of Gdańsk and purified to the specific activity of  $20 \mu\text{mols min}^{-1} \text{ mg}^{-1}$  protein by the method described by Skorkowski & Storey (1987).

### 2.3. Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) was performed according to Laemmli's method (1970), the marker being SDS-7B (Sigma-Aldrich). The samples were subjected to electrophoresis at 20 mA, 25°C for 2.5 h, and the gel was stained with Coomassie Brilliant Blue.

### 2.4. GSH identification

The muscles of brown shrimps *C. crangon* (about 200 mg of the tissue) were homogenized in 1 ml buffer, pH 3.5 (H<sub>2</sub>O : ACN, 90 : 10 v : v, with 1 mM ammonium acetate). After centrifugation (800 g, 5 min) a 100  $\mu\text{l}$  sample

of the supernatant was obtained. The supernatant was adjusted with the buffer, pH 3.5, to a volume of 300  $\mu\text{l}$ .

Linearity was tested using five standards from 0.1 to 10  $\text{mg l}^{-1}$  (0.1, 0.5, 1, 5, 10  $\text{mg l}^{-1}$ ) for GSH.

### 2.5. HPLC-MS analysis

GSH was analysed on a ThermoQuest Finnigan LCQ Deca mass detector equipped with ESI interface (Finnigan, USA). A Kinetex C<sub>18</sub> (100  $\times$  4.6 mm, 2.6  $\mu\text{m}$  100A) column (Phenomenex, Torrance, USA) was used for the chromatographic separation using 50% water (0.04% formic acid) as Solvent A and 50% methanol as Solvent B. The flow rate was 0.3  $\text{ml min}^{-1}$  and 50  $\mu\text{l}$  was injected into the column. Oxidized and reduced glutathione were eluted by isocratic elution chromatography during 6 min. The instrument was run in negative ion mode and in single ion monitoring (SIM) mode (306  $\text{m z}^{-1}$  for GSH). GSH (from Sigma-Aldrich) was used as the analytical standard. The electrospray was held at 5000 V, and the capillary temperature and voltage were set at 350°C and 10 V. The sheath gas (nitrogen) and aux gas were set at 70 and 5 arb. The tube lens offset was 60 V.

### 2.6. Incubation of ME with GSH, BSA and cadmium

The ME stock solution was prepared by exchanging the buffer and removing EDTA (which could interfere with the manganese and cadmium used in the studies reported here) by centrifugation with VivaSpin6.

ME was then diluted in 50 mM Tris-HCl buffer, pH 7.5, to a final ME protein concentration of 0.01  $\text{mg ml}^{-1}$ . ME was preincubated for 30 min with 1 mM or 2 mM GSH, or 5  $\mu\text{g}$  or 20  $\mu\text{g}$  of bovine serum albumin (BSA).

Cadmium chloride (final concentration 1 or 2 mM) was then added and the remaining activity measured after 0, 2, 4, 6, 12, 24 or 48 hrs, as shown in the figure legends. All the incubation experiments were carried out at 4°C. ME activity was tracked spectrophotometrically by observing the appearance of NADPH at 340 nm and 25°C. The standard reaction mixture contained 50 mM Tris-HCl, pH 7.5, 0.5 mM NADP, 5 mM L-malate and 1 mM manganese chloride. Enzyme activities were calculated using  $E \text{ mM} \times 340^{-1} = 6.22$  for NADPH in a 1 cm light-path quartz cell.

### 2.7. Chemicals

Cadmium chloride, glutathione (GSH, GSSG), Tris,  $\text{MnCl}_2$ , albumin (BSA), methanol, acetonitrile, formic acid, acetic acid (all reagents HPLC grade), ammonium acetate and all other chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

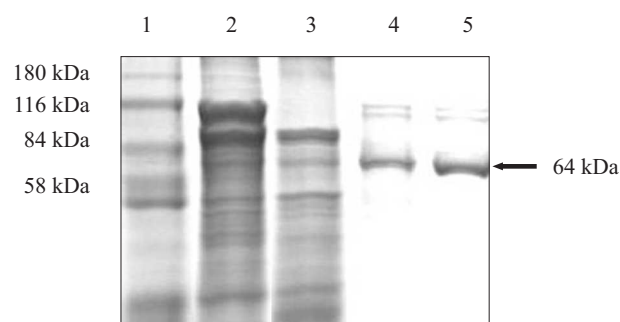
### 3. Results

The results of NADP-ME purification from the abdominal muscle of the brown shrimp (*Crangon crangon*) are presented in Table 1. Shrimp malic enzyme was purified from the abdominal muscle in three chromatographic steps, using a method described earlier, to the specific activity of 20  $\mu\text{mol min}^{-1} \text{mg}^{-1}$  protein (Skorkowski & Storey 1987). Figure 1 shows the SDS-PAGE analysis of protein samples from the different purification steps.

**Table 1.** Purification of NADP-dependent malic enzyme from the abdominal muscle of shrimps *Crangon crangon*

Purification step	Protein [mg]	Specific activity [ $\mu\text{mol min}^{-1} \text{mg}^{-1}$ ]	Total activity [ $\mu\text{mol min}^{-1}$ ]	Yield [%]	Purification (fold)
homogenate	1567.52	0.04	62.70	100	1
DEAE-Sepharose	282.49	0.28	79.10	126	7
2',5'-ADP-Sepharose	5.76	9.75	56.16	89	243
Sepharose 6B	2.60	19.23	50.00	79	480

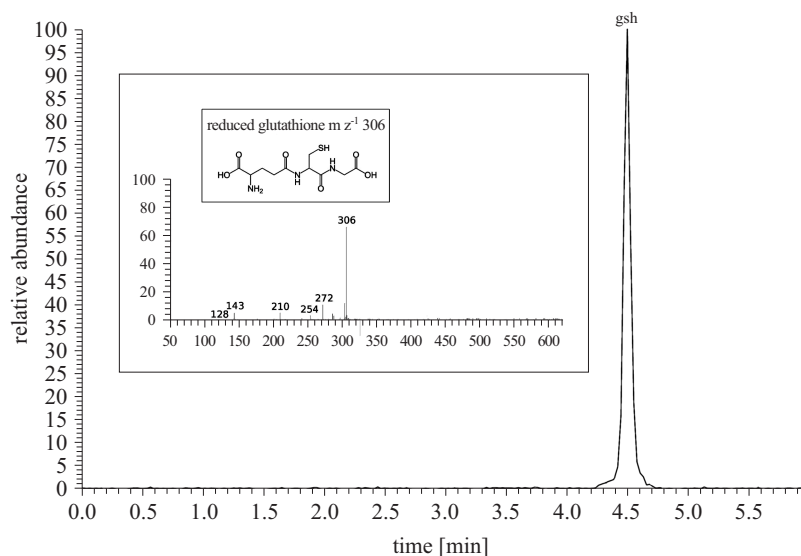
The enzyme was isolated from 80 g of abdominal muscle.



**Figure 1.** SDS-PAGE analysis of protein samples from the abdominal muscle of shrimps *Crangon crangon* from different purification steps after staining with Coomassie Brilliant Blue. Lane 1 – MWM, lane 2 – shrimp abdominal muscle extract, lane 3 – ME after DEAE-Sepharose, lane 4 – ME after 2'5'ADP-Sepharose, lane 5 – ME after Sepharose 6B

The identification of GSH in the abdominal muscle of *C. crangon* inhabiting the Gulf of Gdańsk is presented in Figure 2; the GSH concentration in this muscle was calculated at 5.8 mM (see Table 2).

The effects of a 1 mM cadmium concentration on NADP-dependent ME activity from shrimp abdominal muscle (specific activity 20  $\mu\text{mol}$



**Figure 2.** Representative LC-ESI-MS/MS chromatogram of reduced glutathione ( $T_R$  4.50 GSH) and negative product ion spectra of GSH ( $306 \text{ m z}^{-1}$ )

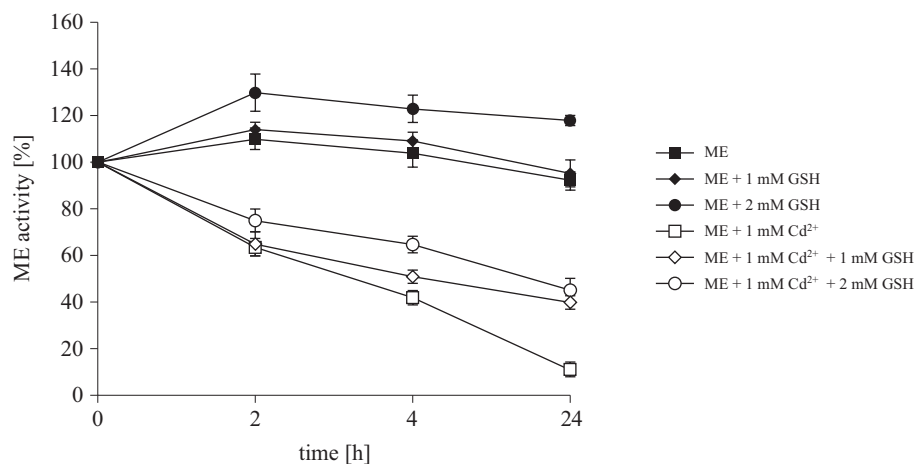
**Table 2.** Reduced glutathione (GSH) content in the abdominal muscle of *Crangon crangon*

Concentration* [ $\text{mg l}^{-1}$ ]	$0.24 \pm 0.02$
Total concentration in abdominal muscle of <i>Crangon crangon</i> [ $\text{mg g}^{-1}$ wet mass]	$1.1 \pm 0.09$
Milimolar concentration in abdominal muscle of <i>Crangon crangon</i> [mM]	$5.8 \pm 0.48$

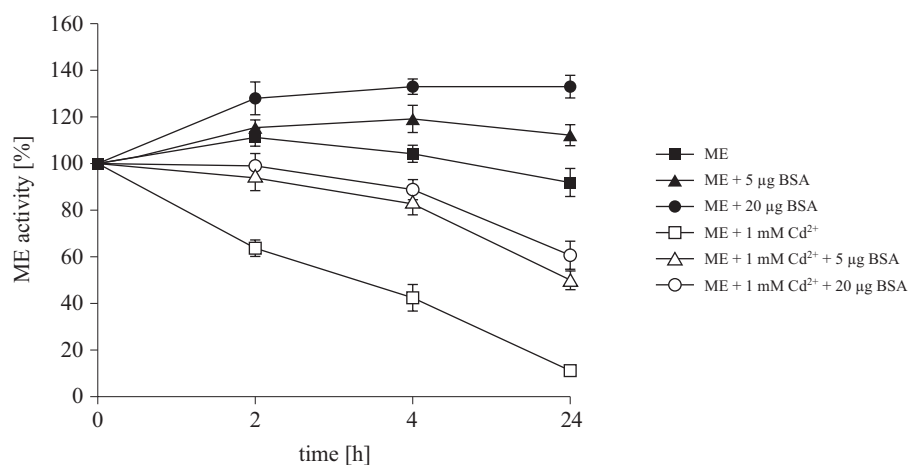
\*Concentration calculated on the basis of the linearity of standard solutions. The values are the means of six assays ( $n = 6$ ).

NADPH  $\text{min}^{-1} \text{ mg}^{-1}$  protein) during 24 hours' exposure in the presence of different GSH concentrations are shown in Figure 3. Cadmium clearly inhibits ME activity, and this inhibition is time-dependent. Incubation for 2 hours caused a ca 50% loss of enzyme activity; after 24 hours this activity had almost completely ceased. The addition of 2 mM GSH to ME increased enzyme activity to 130%. In the presence of different concentrations of GSH, ME was inhibited by cadmium to a far smaller extent, the inhibition being both dose- and time-dependent on GSH concentration (Figure 3).

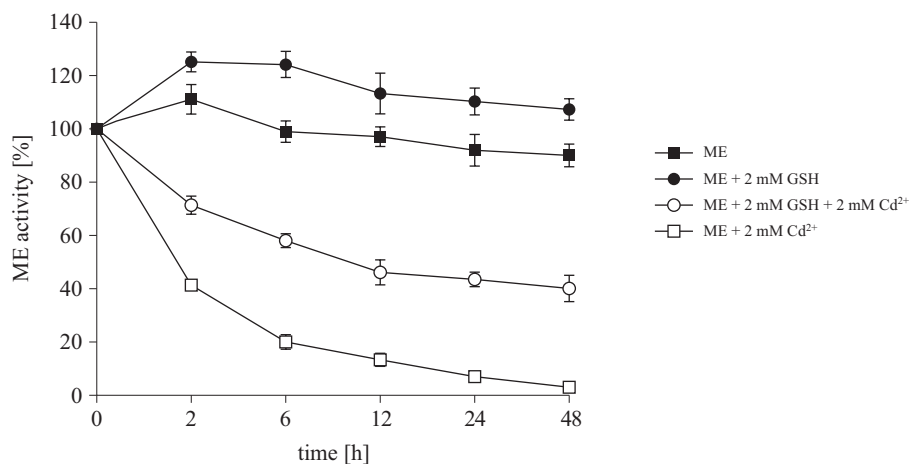
The effect of different concentrations of BSA on ME activity without cadmium and in the presence of 1 mM cadmium during a 24 h incubation are shown in Figure 4. Like GSH, BSA protected ME activity. The addition



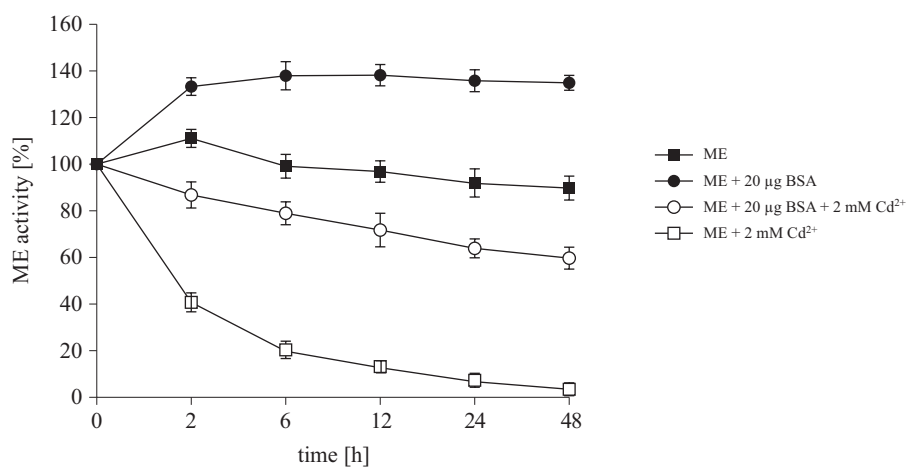
**Figure 3.** Time-dependent inhibition of NADP-dependent malic enzyme activity in the abdominal muscle of *Crangon crangon* by 1 mM cadmium and the protective effect of reduced glutathione (GSH). The reactions were carried out in the presence of 0.5 mM NADP, 5 mM malate, 1 mM Mn<sup>2+</sup>, 50 mM Tris-HCl buffer, pH 7.5 at 25°C and 1 µg of the purified enzyme. GSH was added to the reaction mixtures prior to cadmium at the concentrations as shown in the Figure. The values are the means of three assays



**Figure 4.** Time-dependent inhibition of NADP-dependent malic enzyme activity in the abdominal muscle of *Crangon crangon* by 1 mM cadmium and the protective effect of bovine serum albumin (BSA). The reactions were carried out in the presence of 0.5 mM NADP, 5 mM malate, 1 mM Mn<sup>2+</sup>, 50 mM Tris-HCl buffer, pH 7.5 at 25°C and 1 µg of the purified enzyme. BSA was added to the reaction mixtures prior to cadmium at the concentrations as shown in the Figure. The values are the means of three assays



**Figure 5.** Time-dependent inhibition of NADP-dependent malic enzyme activity in the abdominal muscle of *Crangon crangon* by 2 mM cadmium and the protective effect of reduced glutathione (GSH). The reactions were carried out in the presence of 0.5 mM NADP, 5 mM malate, 1 mM Mn<sup>2+</sup>, 50 mM Tris-HCl buffer, pH 7.5 at 25°C and 1 μg of the purified enzyme. GSH was added to the reaction mixtures prior to cadmium at the concentrations as shown in the Figure. The values are the means of three assays



**Figure 6.** Time-dependent inhibition of NADP-dependent malic enzyme activity in the abdominal muscle of *Crangon crangon* by 1 mM cadmium and the protective effect of bovine serum albumin (BSA). The reactions were carried out in the presence of 0.5 mM NADP, 5 mM malate, 1 mM Mn<sup>2+</sup>, 50 mM Tris-HCl buffer, pH 7.5 at 25°C and 1 μg of the purified enzyme. BSA was added to the reaction mixtures prior to cadmium at the concentrations as shown in the Figure. The values are the means of three assays



of BSA to the incubation medium at a concentration of 20  $\mu\text{g}$  per ml to ME increased enzyme activity to about 130%, as shown for GSH in Figure 3. In the presence of different concentrations of BSA, ME was inhibited by cadmium to a much lesser extent, the inhibition being both dose- and time-dependent on the different concentrations of BSA. BSA is a 70 kDa protein containing about 7% cysteine in an amino acid structure and can protect enzyme activity as a non-specific chaperone (Figure 4).

Figure 5 shows the effect of GSH at 2 mM concentration and in the presence of 2 mM cadmium during a 48-hour incubation with NADP-dependent ME from shrimp abdominal muscle. In the presence of 2 mM of GSH and 2 mM cadmium, the inhibition was time-dependent; GSH can also protect ME activity against higher concentrations of cadmium.

Figure 6 illustrates the effect of 20  $\mu\text{g}$  BSA per ml added to ME during incubation for 48 hours and of 2 mM cadmium on NADP-dependent ME activity from shrimp abdominal muscle. In the presence of 2 mM cadmium, the inhibition was time-dependent; BSA can also protect ME activity against higher concentrations of cadmium (Figure 6).

#### 4. Discussion

Glutathione (GSH) is present in many living systems and often alleviates the adverse effect of xenobiotics, but it is unclear how it affects the inhibition of some enzymes by cadmium (Cd). An intracellular glutathione concentration of up to 8 mM reflects a dynamic balance between reduced glutathione and oxidized glutathione (Griffith 1999). Oxidized glutathione is reduced intracellularly to GSH by glutathione reductase in a NADPH-dependent reaction (Kehrer & Lund 1994). Under physiological conditions and depending on NADPH availability, the GSH/GSSG ratio can reach 100 (Griffith 1999). However, if certain compounds (e.g. malic enzyme, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase) limit the glutathione reductase reaction or NADPH synthesis, oxidized glutathione can accumulate. As shown earlier, the activity of malic enzyme in the abdominal muscle of *Crangon crangon* is about 20 times greater than that of glucose-6-phosphate dehydrogenase. In crustaceans, moreover, both malic enzyme and isocitrate dehydrogenase are more significant as a source of NADPH in somatic muscles (Skorkowski et al. 1980).

The present investigation was undertaken to establish the effects of cadmium on the activity of shrimp muscle ME. The results demonstrate that cadmium inhibits this activity and that glutathione (Figures 3 and 5) and albumin (Figures 4 and 6) can prevent the cadmium-initiated inhibition of malic enzyme. Cadmium can bind to the sulphhydryl groups in proteins

and affect the structure and function of these molecules. MT is a cysteine-rich, metal-binding protein protecting cells from cytotoxic heavy metals, including cadmium, by sequestration (Liu et al. 1995, Waalkes 2000). It was shown earlier that cadmium induces MT in the abdominal muscle of *C. crangon* (Napierka & Radłowska 1998). Cadmium is responsible for several toxic effects that lower the GSH level. In addition, cadmium induces oxidative processes in cells; GSH is the most efficacious antioxidant (Wang et al. 2004). Cadmium complexing by reduced glutathione is one of the first defensive reactions of animals (Bruggeman et al. 1992). GSH is an important intracellular tripeptide containing cysteine (present in cells up to 8 mM) (Griffith 1999). Under normal physiological circumstances, at the expense of NADPH, oxidized glutathione (GSSG) is reduced to GSH by glutathione reductase, leading to a high GSH/GSSG ratio, thereby forming a redox cycle (Canesi & Viarengo 1997, Griffith 1999, Lu 2000, Lee et al. 2008).

Cadmium is an environmental contaminant in seawater that accumulates in organisms; it can be ingested by some animals through their food. It was shown earlier that the cadmium concentration in the abdominal muscles of *C. crangon* inhabiting the Gulf of Gdańsk is 10 times higher than that in the abdominal muscles of shrimps *Palaemon serratus* from Concarneau Bay, Atlantic Ocean (Napierka et al. 1997). The high level of cadmium in *C. crangon* muscles is due to the serious water pollution in the Gulf of Gdańsk, the region from where the shrimps were collected. In fish, cadmium can reach the blood via the alimentary canal, and the albumin present in the blood in high concentrations may act as a chelating agent of cadmium. Albumin is a 70 kDa protein containing about 7% cysteine in its amino acid sequence and can act as non-specific chaperone to some enzyme activity. In this way, albumin can protect other protein molecules from direct cadmium binding, as has been shown for malic enzyme (Figure 4 and 6).

ME catalyses the reversible decarboxylation of malate to form pyruvate in the presence of NADP coenzyme: the divalent manganese or magnesium cation is necessary to start the enzymatic reaction. Over the pH range 6.5–7.0, the rate of pyruvate carboxylation is equal to the rate of malate decarboxylation, suggesting an anaplerotic function for abdominal muscle ME in *C. crangon* (Biegniewska & Skorkowski 1983). ME activity in crustacean and fish muscles is much higher than that observed in most terrestrial species (Skorkowski 1988). ME is particularly interesting since it uses pyruvate as a substrate and provides an alternative route for pyruvate metabolism in fish muscle during the active mobilization of protein as an energy source or supports gluconeogenesis in the liver during salmon spawning migration (Mommsen et al. 1980, Mommsen 2004).

Our previous study showed that ME is a molecule highly sensitive to cadmium exposure and that this strongly inhibits ME activity (Biegniewska et al. 1993). In a cation combination added in vitro to the incubation medium, cadmium inhibits enzyme activity down to the value this would have if cadmium were added alone. In the presence of both cations (cadmium and manganese), manganese does not activate ME activity (Biegniewska et al. 1993). Inhibition of ME activity by cadmium, and in consequence the decreasing formation of NADPH, could interfere with the cellular mechanism against detoxification and oxidative stress. This study showed that the toxic effect on malic enzyme activity of cadmium, used in higher concentrations than are present in shrimp muscles, could be counteracted by lower glutathione and albumin concentrations than are present in fish. Glutathione and albumin can protect marine animals against pollution by toxic cadmium. The results of the present work suggest that endogenous cellular glutathione reduces the Cd inhibition of NADP-dependent malic enzyme, thus protecting it; this enzyme could therefore increase NADPH formation.

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