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Dual control of cytosolic metals by lysosomal transporters in lobster hepatopancreas

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SUMMARY

This study describes the membrane transport mechanisms used by lobster (*Homarus americanus)* hepatopancreatic epithelial lysosomes to accumulate and sequester heavy metals from the cytosol, and thereby aid in the regulation of these ions entering the animal from dietary constituents. The present investigation extends previous work describing lysosomal metal uptake by cation exchange with protons and suggests that a second, parallel, lysosomal transport process involving metal–thiol conjugates may work in conjunction with the cation antiporter to control cytoplasmic metal concentrations. Transport of $^{65}Zn^{2+}$ by lysosomal membrane vesicles (LMV) incubated in 1 mmol l^{-1} glutathione (GSH) was not significantly different from metal transport in the absence of the tripeptide. However, preloading LMV with 1 mmol l^{-1} α-ketoglutarate (AKG), and then incubating in a medium containing 1 mmol l^{-1} GSH, more than doubled metal uptake, compared with vesicles equilibrated with chloride or possessing an outwardly directed chloride gradient. Kinetic analysis of lysosomal $^{65}Zn^{2+}$ influx as a function of zinc concentration, in vesicles containing 1 mmol l^{-1} AKG and incubated in 1 mmol l^{-1} GSH, revealed the presence of a sigmoidal, low affinity, high capacity carrier process transporting the metal into the organelle. These data indicated the possible presence of an organic anion exchanger in lobster lysosomal membranes. Western blot analysis of LMV with a rabbit anti-rat OAT1 antibody showed the presence of an orthologous OAT1-like protein (approximate molecular mass of 80 kDa) signal from these membranes. These results, and those published previously, suggest the occurrence of two metal transporters on hepatopancreatic membranes, a high affinity, low capacity cation antiporter and a low affinity, high capacity organic anion exchanger. Together these two systems have the potential to regulate cytoplasmic metals over a wide concentration range.

Key words: zinc transport, lysosomes, detoxification, organic anion transport, OAT1, glutathione, α-ketoglutarate, heavy metals, lobster, *Homarus americanus*, hepatopancreas.

INTRODUCTION

Many marine invertebrates live in environments where they are continuously exposed to metals through either the water or the food. Some of these metals are important nutritional elements such as iron, copper and zinc, and are used biochemically as regulatory factors for a significant number of proteins (Watanabe et al., 1997; Beyersmann and Haase, 2001; Klasassen, 2001). Other metals such as cadmium, lead and mercury have no biological role and, when present in biological systems, interfere with normal physiological activities and can lead to pathological conditions. All metals in excess can be deleterious to cells and tissues and, therefore, their circulating concentrations must be tightly regulated.

The crustacean hepatopancreas is a multifunctional organ that is composed of hundreds of blind-ending tubules lined with four types of epithelial cell that together perform many functions of the mammal intestine, pancreas and liver (Van Weel, 1974; Gibson and Barker, 1979; Paquet et al., 1993; Verri et al., 2001). The hepatopancreas is a site of heavy metal detoxification, a process maintaining low cytosolic metal concentrations. This process involves the thiol-containing, metal-binding proteins, glutathione and metallothionein, that are up-regulated by metal exposure (Brouwer et al., 1992; Brouwer et al., 1995), and several organelles such as mitochondria (Chavez-Crooker et al., 2002), endoplasmic reticulum (Mandal et al., 2005) and lysosomes (Chavez-Crooker et al., 2003; Mandal et al., 2006; Sterling et al., 2007).

Lysosomes from vertebrates and crustaceans are centers of calcium and metal sequestration that have an acidic interior as a result of proton-transporting activities of ATP-dependent, bafilomycin-sensitive, V-ATPases localized on their membranes (Pisoni and Thoene, 1991; Chou et al., 1992; Chavez-Crooker et al., 2003). These ATPases not only create an acidic interior but also impose a membrane potential across their membranes, inside positive. In crustaceans, the membrane potential and accumulated lysosomal protons are used as driving forces by an electrogenic 3 H^+/1 $cation^{2+}$ antiporter for divalent metal and calcium uptake from the cysotol (Mandal et al., 2006; Sterling et al., 2007). Once in the lysosomes, the metals form a complex with polyvalent anions, such as sulfate or phosphate, forming an impermeable precipitate that sequesters the metal and effectively detoxifies it (Hopkin, 1989).

Several studies examining the nature of the substance inside invertebrate epithelial lysosomes have described the presence of high concentrations of thiol-containing organic material, and the amino acid composition of this material appeared similar to that previously characterized in cytosolic metallothioneins (Viarengo et al., 1985a; Viarengo et al., 1985b; Viarengo et al., 1989). These findings suggest that thiol agents bind metals in the cytoplasm and are transferred to the lysosomes for detoxification. However, in these invertebrates, the nature of the physiological process by which these thiol-containing elements entered lysosomes from the cytosol was not described. Glutathione (GSH) is a tripeptide composed of glutamate, cysteine and glycine moieties and is one of the thiol-containing, low molecular weight proteins up-regulated by an increase in cytosolic metal content (Viarengo, 1994; Burlando et al., 1997). It has been demonstrated that GSH can form complexes with different metals, such as Cu^{2+}, Cd^{2+} and Hg^{2+}, and that these complexes may move into lysosomes (Viarengo, 1994). The present investigation

examines a transport mechanism for GSH and metal uptake into lysosomal membrane vesicles from the epithelial cytosol and its potential for contributing to the cytoplasmic regulation of these potentially toxic cations.

MATERIALS AND METHODS

Lysosomal membrane vesicle isolation

Hepatopancreatic lysosomal membrane vesicles (LMV) were prepared as previously described using an isotonic Percoll gradient and methionine methyl ester (Chavez-Crooker et al., 2003). LMV were prepared from live male Atlantic lobster (*Homarus americanus* Milne-Edwards 1837) purchased from a local commercial dealer in Jacksonville, FL, and maintained at 15°C. Hepatopancreas tissue was placed in cold buffer A (mmol l^{-1}: 250 sucrose, 20 Hepes, 1.0 EDTA, 0.1 PMSF at pH 7.0 with Tris base). The tissue was homogenized in buffer A and diluted 10-fold in the buffer. The homogenate was centrifuged at 800 ***g*** for 10 min (this centrifugation was performed twice). The resulting supernatant was centrifuged at 20,000 ***g*** for 10 min. The resulting pellet was diluted in buffer B (mmol l^{-1}: 250 sucrose, 20 Hepes at pH 7.0 with Tris base). The suspension was then mixed with isotonic Percoll in a 9:11 ratio (pellet in buffer B:isotonic Percoll) and centrifuged at 40,000 ***g*** for 90 min. The brown dense lysosomal band near the bottom of the gradient was removed, diluted with buffer B, and centrifuged at 20,000 ***g*** for 10 min. The pellet was then incubated in a freshly prepared buffer B containing 5 mmol l^{-1} methionine methyl ester, 2 mg ml^{-1} bovine serum albumin and 2 mmol l^{-1} $MgCl_2$ for 20 min. An equal volume of ice-cold isotonic Percoll was added and the incubation mixture was centrifuged at 35,000 ***g*** for 30 min. The purified LMV (brown band at the top of the gradient) were then washed with the appropriate loading buffer several times to remove isotonic Percoll solution. The final LMV concentration was determined using a Bradford protein assay and vesicles were diluted to 1 mg ml^{-1} in an appropriate loading buffer. Vesicles in loading buffer were passed through a 22 gauge needle and incubated for approximately 2 h on ice before use in the uptake assays.

Transport studies

The purified LMV from the hepatopancreas of single animals were used in isotope experiments to determine the characteristics of ^{65}Zn uptake under a variety of incubation medium conditions. $^{65}ZnCl_2$ was obtained from Oak Ridge National Laboratory, TN (specific activity, 0.0303 μCi μg^{-1}). Experiments were initiated by dispensing 20 μl of LMV suspension loaded with appropriate inside buffer into 180 μl of appropriate outside buffer containing ^{65}Zn and unlabelled $ZnSO_4$. The reaction was stopped by adding 3 ml of ice-cold stop solution consisting of the appropriate inside buffer, and tubes were vacuum-filtered using the Millipore™ filtration methods of Hopfer and colleagues (Hopfer et al., 1973). Filters were placed in vials with 5 ml of scintillation fluid for radioactivity assessment in a Beckman Coulter LS 6500 multi-purpose scintillation counter.

Non-specific isotope binding was measured by rapidly filtering 20 μl of LMV suspension at the lip of a test tube containing 3 ml stop solution and outside media containing ^{65}Zn. Non-specific isotope binding was subtracted from the total isotope measurement, and time courses and kinetics are presented as means of 3–5 replicates per point with corresponding standard errors. Experiments were repeated 2–3 times with additional lobsters with repetitions providing qualitatively similar results. The time course and kinetics of isotope uptake into LMV and the resulting data were analyzed and graphed using Sigma Plot 10.0 software.

Immunoblotting

Samples of 20 or 50 μg of *H. americanus* hepatopancreas crude homogenate, pre-methionine methyl ester-incubated LMV, purified LMV, and rat small intestine proteins were separated by SDS-PAGE. The proteins were transferred to PVDF membrane in 10 mmol l^{-1} CAPS buffer at pH 11 and 10% methanol at 75 V for 25 min. The membranes were blocked for a minimum of 1 h with 5% non-fat dry milk in PBS and 0.05% Tween-20. The blocked membranes were probed for at least 1 h with rabbit anti-rat polyclonal OAT1 antibody. The blots were washed of primary antibody and incubated with goat anti-rabbit IgG (H+L), peroxidase-conjugated secondary antibody. The blots were washed of secondary antibody and incubated in Supersignal West Pic Chemiluminescent substrate and then exposed to X-ray film for varying times.

RESULTS

Effect of external GSH on $^{65}Zn^{2+}$ uptake by LMV

$^{65}Zn^{2+}$ (20 μmol l^{-1}) uptake by hepatopancreatic LMV in the absence of external GSH rapidly reached equilibrium at approximately 4 nmol mg^{-1} protein by 10 min (Fig. 1). Addition of 1 mmol l^{-1} GSH to the external incubation medium did not significantly (P>0.05) affect either the initial rate of $^{65}Zn^{2+}$ accumulation at 5 min incubation or the apparent equilibrium attained by the metal by 60 min of incubation.

Effect of preloaded anions on $^{65}Zn^{2+}$ uptake by LMV

In hepatopancreatic LMV an outwardly directed chloride gradient stimulated the uptake of both sulfate and oxalate and the suggestion was made that an anion exchanger was present on these membranes which facilitated the antiport of both inorganic and organic anions (Sterling et al., 2007). In order to see whether $^{65}Zn^{2+}$, complexed with GSH, could use this anion exchanger, an experiment was designed to compare the uptake by LMV of $^{65}Zn^{2+}$ in the presence of 1 mmol l^{-1} GSH, and with an outwardly directed Cl^- gradient, with equal Cl^- concentrations on the two sides of the LMV, and with a 1 mmol l^{-1} outwardly directed α-ketoglutarate (AKG) gradient. The last treatment would also have 0.1 mmol l^{-1} AKG

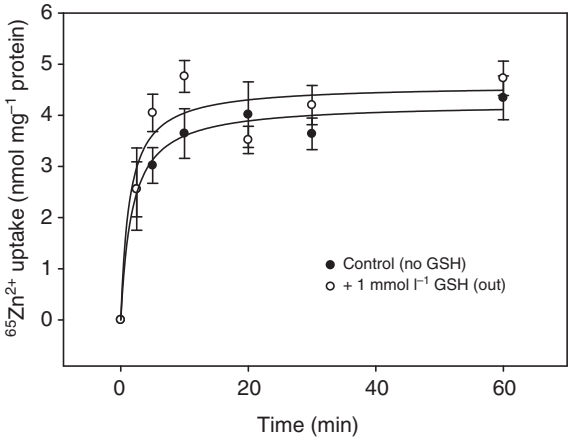

Fig. 1. Effect of 1 mmol l^{-1} glutathione (GSH) on 20 μmol l^{-1} $^{65}Zn^{2+}$ uptake by hepatopancreatic lysosomal membrane vesicles (LMV). Vesicles were loaded with 150 mmol l^{-1} mannitol and 20 mmol l^{-1} Hepes, at pH 7.0. The outside control medium contained 20 μmol l^{-1} $ZnSO_4$, 150 mmol l^{-1} mannitol and 20 mmol l^{-1} Hepes. The experimental medium consisted of the control medium with the addition of 1 mmol l^{-1} reduced GSH and 2 mmol l^{-1} DTT. Symbols are means (N=3 samples per point from a single LMV preparation) ± 1 s.e.m. The experiment was repeated twice with separate animals with qualitatively similar results.

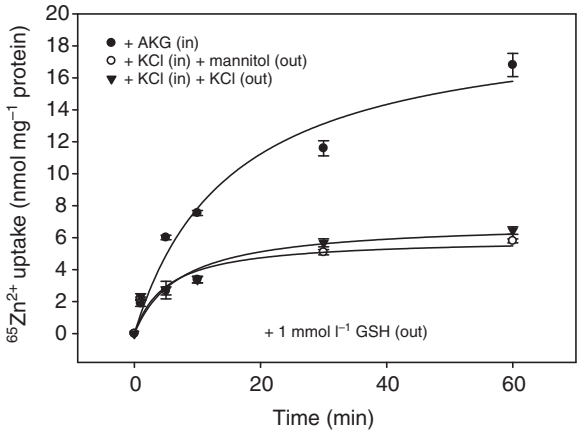

Fig. 2. Effects of outwardly directed anion gradients on 1 mmol l^{-1} GSH-stimulated 20 μmol l^{-1} $^{65}Zn^{2+}$ uptake by hepatopancreatic LMV. Vesicles were prepared to have an outwardly directed 25 mmol l^{-1} Cl^- gradient (inside buffer=25 mmol l^{-1} KCl, 100 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris, pH 7.0; outside buffer=150 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris, 1 mmol l^{-1} GSH, 2 mmol l^{-1} DTT, pH 7.0, 20 μmol l^{-1} $^{65}ZnSO_4$); equal 25 mmol l^{-1} Cl^- concentrations on each side of the LMV (inside and outside buffers=25 mmol l^{-1} KCl, 100 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris, pH 7.0, plus 1 mmol l^{-1} GSH, 2 mmol l^{-1} DTT and 20 μmol l^{-1} $^{65}ZnSO_4$ outside); or a 1 mmol l^{-1} outwardly directed α-ketoglutarate (AKG) gradient (inside buffer=150 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris, 1 mmol l^{-1} AKG, pH.7; outside buffer=150 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris, 1 mmol l^{-1} reduced GSH, 2 mmol l^{-1} DTT, pH 7.0, 20 μmol l^{-1} $^{65}ZnSO_4$). Vesicles loaded with 1 mmol l^{-1} AKG would also have 0.1 mmol l^{-1} AKG carried over into the external medium resulting from the incubation protocol used. Symbols are means (*N*=3 samples per point from a single LMV preparation) ± 1 s.e.m. The experiment was repeated with separate lobsters twice with qualitatively similar results.

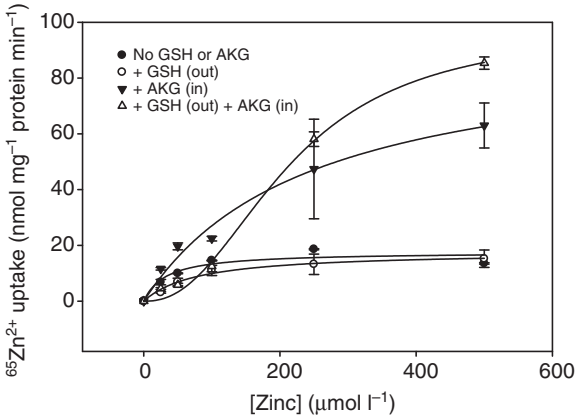

Fig. 3. Effects of 1 mmol l^{-1} GSH + 0.1 mmol l^{-1} AKG outside and 1 mmol l^{-1} AKG inside on 2.5 min influxes of $^{65}Zn^{2+}$ (25–500 μmol l^{-1} as $ZnSO_4$) by hepatopancreatic LMV. Vesicles were loaded and incubated in media of similar composition to those in Fig. 2, except without KCl. Control influx (no GSH or AKG) was hyperbolic and not significantly different (*P*>0.05) from influx measured with only an inwardly directed 1 mmol l^{-1} GSH gradient. Influx measured in the presence of an outwardly directed 1 mmol l^{-1} AKG gradient was hyperbolic, but significantly (*P*<0.01) greater than control influx. Influx with 1 mmol l^{-1} GSH outside, 1 mmol l^{-1} AKG inside and 0.1 mmol l^{-1} AKG outside was sigmoidal, exhibiting a Hill coefficient greater than 2, suggesting cooperative binding of metal ions to the exchanger or GSH molecules under these conditions. Vesicles loaded with 1 mmol l^{-1} AKG would also have 0.1 mmol l^{-1} AKG carried over into the external medium due to the incubation protocol used. Symbols are means (*N*=3–5 samples per point from a single LMV preparation) ± 1 s.e.m. The experiment was repeated with separate lobsters twice with qualitatively similar results.

present on the outside of the LMV due to a 10% carry-over of vesicular contents to the external medium in the transport protocol used in these experiments. Fig. 2 shows that $^{65}Zn^{2+}$ uptake was the same in vesicles possessing an outwardly directed chloride gradient as it was in vesicles exhibiting equal chloride concentrations on the two sides of the membrane. However, vesicles with an outwardly directed AKG gradient (0.1 mmol l^{-1} AKG outside) demonstrated both a significantly greater initial rate of uptake and a higher equilibrium value of zinc accumulation than did either of the chloride treatments. These results suggest the possible presence of a second anion exchange mechanism on lysosomal membranes that appears to prefer coupled transport of zinc with organic anions.

Kinetics of zinc influx by LMV in the presence and absence of GSH and AKG

$^{65}Zn^{2+}$ (25–500 μmol l^{-1}) influx (2.5 min incubations) was measured in the presence and absence of external 1 mmol l^{-1} GSH and internal 1 mmol l^{-1} AKG (0.1 mmol l^{-1} AKG outside) (Fig. 3). As displayed in this figure, $^{65}Zn^{2+}$ influx in both the presence and absence of external 1 mmol l^{-1} GSH was a hyperbolic function of external zinc concentration and followed the Michaelis–Menten equation for carrier-mediated transport:

$$J_{Zn} = J_{max}\,[Zn] / (K_m + [Zn])\,, \quad (1)$$

where J_{Zn} is zinc influx at each of the zinc concentrations investigated, J_{max} is the apparent maximal influx rate, K_m is the apparent affinity constant and [Zn] is the concentration of zinc. Values for the kinetic constants K_m and J_{max} obtained from Fig. 3 are shown in Table 1. These results suggest that the addition of 1 mmol l^{-1} GSH to the external medium bathing LMV had only a slight effect on the entry of $^{65}Zn^{2+}$ compared with the treatment lacking the thiol agent. Addition of 1 mmol l^{-1} AKG to the inside of LMV (0.1 mmol l^{-1} AKG outside) incubated in a medium lacking 1 mmol l^{-1} GSH resulted in a significantly elevated hyperbolic influx curve compared with those described above for the treatments lacking the internal organic anion. The J_{max} for zinc influx in the presence of AKG was 5 times that in its absence, while the K_m value was raised approximately 7 times under these conditions (Table 1). These results suggest that the enhanced hyperbolic zinc uptake in AKG-loaded vesicles was likely due to $^{65}Zn^{2+}$ binding to the carry-over of 0.1 mmol l^{-1} AKG in the outside medium and its homoexchange with internal AKG.

Influx of $^{65}Zn^{2+}$ in LMV in the presence of 1 mmol l^{-1} GSH outside and 1 mmol l^{-1} AKG inside (0.1 mmol l^{-1} AKG outside) was a sigmoidal function of external zinc concentration (Fig. 3) and followed the Hill equation for multisite cooperativity:

$$J_{Zn} = J_{max}\,[Zn]^n / ([Zn]^n{}_{0.5} + [Zn]^n)\,, \quad (2)$$

where n is the Hill coefficient and defines the extent of multisite cooperativity that occurred for zinc binding to the transporter, and is an approximation of the number of zinc ions being transported for each transport event, $[Zn]^n{}_{0.5}$ (Segel, 1975) is the apparent affinity constant modified for cooperative interactions between binding zinc ions, J_{max} is the maximal transport velocity of zinc transport across

Table 1. Effects of GSH and AKG on $^{65}Zn^{2+}$ influx kinetic constants in LMV

Influx conditions	K_m or $[Zn]^n_{0.5}$ (μmol l^{-1} Zn)	J_{max} (nmol mg^{-1} min^{-1})	n
No GSH or AKG (control)	32±6[a]	17±2[a]	n.d.
+ GSH (outside)	79±15[b]	17±1[a]	n.d.
+ AKG (inside)	244±64[c]	93±11[b]	n.d.
+ GSH (outside + AKG (inside)	214±22[c]	97±8[b]	2.3±0.3

LMV, lysosomal membrane vesicles; n.d., not determined; K_m, apparent affinity constant; $[Zn]^n_{0.5}$, apparent affinity constant modified for cooperative interactions between binding zinc ions; J_{max}, maximal transport velocity of zinc transport across the vesicular membranes; n, Hill coefficient.

Values in table are means ±1 s.e.m. Kinetic constants were determined on 3–5 replicates per condition and graphical analysis was conducted with Sigma Plot 10.0 software.

Statistically significant differences (P<0.05) between treatments were determined with ANOVA and are indicated by different superscript letters.

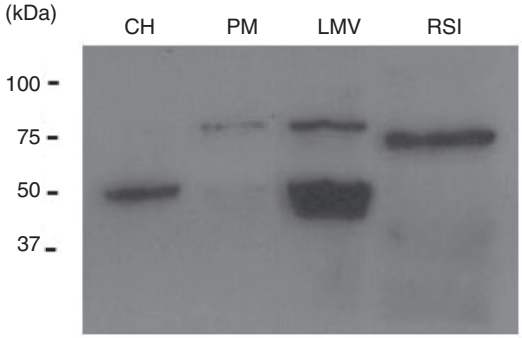

Fig. 4. Western blot of lobster LMV using rabbit anti-rat OAT1 antibody. Lane 1 was loaded with crude homogenate (CH). Lane 2 was loaded with the cellular fraction prior to addition of methionine methyl ester (PM). Lane 3 was loaded with lysosome membrane vesicles (LMV). Lane 4 was loaded with rat small intestine homogenate (RSI) which served as the control at 80 kDa.

the vesicular membranes, and $[Zn]^n$ is the external zinc concentration. The kinetic coefficients in Table 1 indicate that hyperbolic $^{65}Zn^{2+}$ influx by LMV loaded with 1 mmol l^{-1} AKG (0.1 mmol l^{-1} AKG outside), but not incubated in 1 mmol l^{-1} GSH outside, displayed similar K_m ($[Zn]^n_{0.5}$) and J_{max} values to those obtained by sigmoidal entry of the metal when AKG was inside (0.1 mmol l^{-1} AKG outside) and GSH was outside. Differences in the shapes of the respective influx curves (hyperbolic *vs* sigmoidal) were therefore likely a function of the number of zinc ions being transported across the membrane in the two treatments. Hyperbolic influx with internal AKG (0.1 mmol l^{-1} AKG outside) appears to reflect the transport of a single metal ion with external 0.1 mmol l^{-1} AKG during homoexchange transport events, while the sigmoidal nature of the influx when both GSH and AKG were present, and the Hill coefficient was between 2 and 3, suggests a transport process involving multiple ions and an undetermined interaction between external AKG (0.1 mmol l^{-1}) and external GSH (1 mmol l^{-1}).

The results in Fig. 3 suggest that two carrier-mediated zinc transport systems may be present on lysosomal membranes, one accommodating the metal at low intracellular concentrations (low K_m value) and the other effective at considerably higher cytoplasmic metal levels (high K_m or $[Zn]^n_{0.05}$ values).

Western blot analysis of possible lysosomal zinc transporter

The OAT1 (organic anion transporter) is an organic anion exchanger that has been identified in mammals as a heavy metal transport system that transports thiol conjugates of metals (Zalups and Barfuss, 2002). In order to see whether an ortholog of the mammalian OAT1 transporter might be present in hepatopancreatic lysosomal membranes, and be responsible for the exchange of GSH-Zn^{2+}/AKG as described by the sigmoidal influx of metal in Fig. 3, western blot analysis was carried out on LMV using a rabbit anti-rat OAT1 antibody. The results in Fig. 4 show the presence of an orthologous OAT1-like protein (approximate molecular mass of 80 kDa) signal from lobster LMV. A cross-reacting signal from LMV of approximately 50 kDa was also observed which is believed to correspond to the un-glycosylated form of the lobster OAT1-like protein. While these results are suggestive of an OAT1-like transport system in hepatopancreatic LMV, these data must be considered tentative because of the use of a mammalian, rather than a more specific lobster, antibody in the western blot analysis. However, these physiological and western blot data do provide strong support for an organic anion exchanger in lobster LMV.

DISCUSSION

Epithelial lysosomes of invertebrates have long been known to be sites of molecular degradation and xenobiotic sequestration and detoxification (Al-Mohanna and Nott, 1985; Al-Mohanna and Nott, 1987; Al-Mohanna and Nott, 1989; Hopkin, 1989; Viarengo et al., 1989; Sokolova et al., 2005). A variety of lysosomal hydrolytic enzymes, effective at acidic pH, play important molecular recycling roles for many organic compounds such as proteins and small peptides (Pisoni and Thoene, 1991; Chou et al., 1992; Thamotharan et al., 1997). These organelles are also effective depots of environmental heavy metals that enter animals through the gastrointestinal tract and across other epithelial-lined structures such as the gills. Lysosomal storage of metals occurs in epithelial cells of most invertebrate phyla and the processes involved in this storage serve to lower circulating concentrations of these materials in the hemolymph which might otherwise impair sensitive tissues such as nerves and muscles or interfere with the precision of nuclear genetic replication. While information regarding the ubiquity of invertebrate metal storage in lysosomes is widely available (Hopkin, 1989), the physiological transport mechanisms by which these organelles transfer potentially toxic metals from the epithelial cytosol to the organelle interior are not readily appreciated.

Lobster (*H. americanus*) hepatopancreatic epithelial lysosomes transport the metals zinc, copper and cadmium from cytoplasm to organelle interior by a shared carrier process that exchanges metals for intra-organelle hydrogen ions (Chavez-Crooker et al., 2003; Mandal et al., 2006; Sterling et al., 2007). Protons for the antiporter are derived from the activities of an ATP-dependent, bafilomycin-sensitive, membrane V-ATPase creating an acidic organelle interior (Chavez-Crooker et al., 2003). The exchange stoichiometry for the cation antiporter is 3 H^+/1 Zn^{2+} (1 Cu^{2+}, 1 Cd^{2+}) (Sterling et al., 2007), and metal uptake is decreased in the presence of bafilomycin (Chavez-Crooker et al., 2003). Lysosomal vesicles with an induced inside positive membrane potential (K^+/valinomycin) and acidic pH exhibited a greater metal uptake than did short-circuited vesicles or those with an imposed inside negative potential (Sterling et al., 2007). These results suggest that

in vivo lysosomes are able to accumulate cytoplasmic divalent metals in exchange for organelle protons using ATP to create a hydrogen ion gradient and induced membrane potential to power the cation exchange process. Once inside lysosomes cationic metals appear to precipitate with polyvalent anions, such as sulfate and phosphate, that may be independently transported from cytosol to vesicular interior by a similar electrogenic anion antiporter that exchanges polyvalent anions with monovalent anions (e.g. hydroxyl and chloride ions) (Sterling et al., 2007). The resulting lysosomal concretion, involving complexed metals and anions, may grow in size during the life of the cell and finally be extruded into the gastrointestinal lumen for evacuation with the feces. These combined processes provide a regulatory mechanism for controlling the amount of cytoplasmic metal that may be available for transfer to the blood across the epithelial basolateral plasma membrane.

The results of the present investigation suggest that there may be a second, independent, lysosomal membrane transport protein involved in regulating cytoplasmic metal concentrations by facilitating their transfer to the organelle interior. In mammal renal basolateral plasma membranes an organic anion transporter (OAT) exchanges organic anions across this cell pole, thereby regulating blood concentrations of these metabolites. The thiol agent GSH readily complexes with divalent metals such as mercury, cadmium or zinc and uses the mammalian OAT1 transport protein to clear the blood of these metals by exchange with cytoplasmic AKG (e.g. GSH-Zn/AKG) or other cytoplasmic organic anions (Zalups and Barfuss, 2002). The results of the present investigation suggest that uptake of $^{65}Zn^{2+}$ by lobster hepatopancreatic lysosomal membrane vesicles is greater from an incubation medium containing GSH and with preloaded AKG than with chloride (Fig. 2), supporting the involvement of an organic anion exchanger in metal accumulation. Additionally, the western blot shown in Fig. 4 suggests the possible presence of an OAT1-like ortholog in hepatopancreatic lysosomal membranes that may be the transporter responsible for the uptake of $^{65}Zn^{2+}$ linked to GSH, although these results would have to be confirmed with a lobster-specific antibody. Finally, $^{65}Zn^{2+}$ influx kinetics by AKG-preloaded vesicles in the presence and absence of external GSH, displayed in Fig. 3, suggest that two carrier-mediated transport processes co-exist on hepatopancreatic lysosomal membranes and display markedly different kinetic constants (Table 1). The high affinity system is likely the 3 H^+/1 Zn^{2+} exchanger previously characterized (Mandal et al., 2006; Sterling et al., 2007), while the low affinity carrier process appears to be an organic anion exchanger. As indicated in Table 1, the high affinity, hyperbolic, antiporter has an apparent binding affinity for zinc (i.e. 32±6 μmol l^{-1}) which is 7 times higher than that displayed by the low affinity, sigmoidal, Zn-GSH/AKG exchanger (i.e. 214±22 μmol l^{-1}). These apparent affinity dissimilarities would suggest that the two different carriers may function at different ends of the cytoplasmic metal concentration range. In addition, the sigmoidal nature and a Hill coefficient for the organic anion exchanger of between 2 and 3 suggest that at high cytoplasmic metal concentrations this carrier process may be transporting multiple metal ions into lysosomes, effectively clearing the cytosol of these elements at an accelerated rate. The results from Fig. 3 suggest that both external AKG and GSH, each linked to zinc, may be able to enter AKG-loaded vesicles using the same anion exchanger by either homoexchange or heteroexchange. Differences in influx curve shape (hyperbolic *vs* sigmoidal) in Fig. 3 appear to reflect which external substrate is exchanging with preloaded AKG and the relative number of zinc ions associated with it. If external AKG and GSH are using the same anion exchanger to facilitate the uptake of zinc, the curves in Fig. 3 suggest that the transporter has a higher apparent binding affinity for external AKG and transports it and a single zinc ion into vesicles at lower zinc concentrations, while the transport of GSH-Zn appears to take place at higher metal concentrations and transfers 2 or more metal ions during each transport cycle (Hill coefficient=2.3±0.3; Table1).

Fig. 5 is a working model of a proposed dual control mechanism for the regulation of cytoplasmic heavy metal concentration by epithelial lysosomal membranes based on results from the present investigation and those presented previously (Chavez-Crooker et al., 2003; Mandal et al., 2006; Sterling et al., 2007). As indicated in this model, cytosolic metal concentration may be regulated over a wide concentration range, as might occur during normal 'housekeeping' activities, when metal concentrations may be low, or during temporary periods of increased metal accumulation from contaminated dietary constituents. During periods of low metal exposure, the high affinity, electrogenic, cation antiporter (protein 1) may function to keep metals in the cytoplasm at low concentrations. When cytosolic metals increase in concentration beyond the high affinity of the cation exchanger, GSH and/or AKG may be upregulated through metal-induced metabolic activities (Thomas and Wofford, 1984; Thomas and Juedes, 1992; Viarengo, 1994; Lash and Zalups, 1998) and provide a low affinity chelator to bind metals and transfer them to the organelle interior *via* the Zn-GSH/AKG conjugate exchanger (protein 2). Through the combined activities of these two exchangers, cytoplasmic metal concentrations may be regulated within a narrow range that is appropriate for enzymatic requirements or for transfer to the blood for systemic circulation.

Fig. 6 illustrates how the two carrier processes proposed in Fig. 5 may work together to control the total uptake of zinc from the epithelial cytosol. Data in this figure were calculated using the K_m

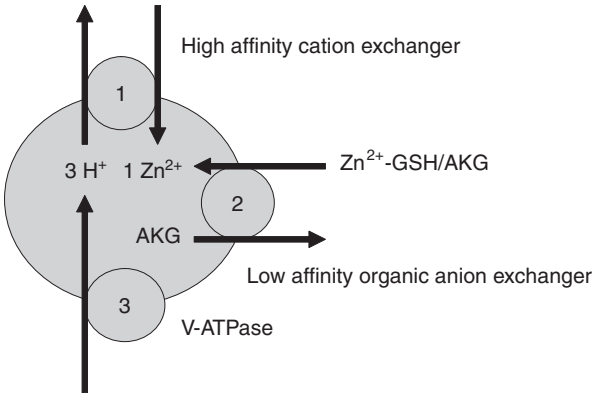

Fig. 5. Working model for the dual control of cytoplasmic metal concentration by lysosomal membrane transporters. Previous studies identified an electrogenic 3 H^+/1 Zn^{2+} antiporter (protein 1) in lysosomal membranes that was powered by an ATP-dependent V-ATPase (protein 3), creating an acidic vesicular interior with an electrically positive internal potential (Mandel et al., 2006; Sterling et al., 2007). The present investigation suggests that a parallel organic anion exchanger (protein 2) is also on this membrane and exchanges an intravesicular organic anion such as AKG for a zinc ion conjugated to external AKG or a thiol-containing agent such as GSH. Because the cation antiporter is a high affinity, low capacity transporter, while the anion exchanger is a low affinity, high capacity carrier, in combination the two transporters would be capable of transporting metals from cytoplasm to lysosomal interior over a wide metal concentration range.

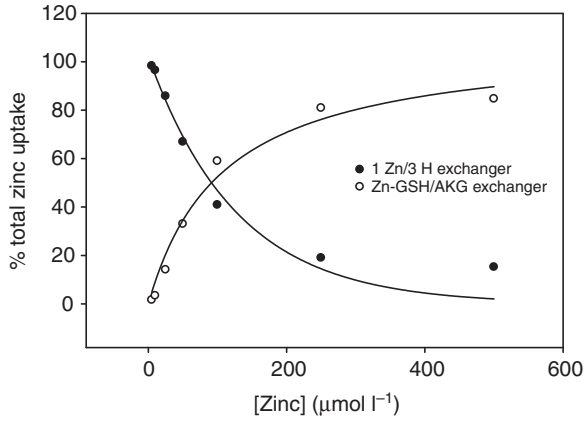

Fig. 6. Relative contribution to total metal uptake from epithelial cytoplasm by each metal transporter described in this report. At low cytosolic metal concentrations around 10–20 μmol l^{-1}, the cation antiporter would contribute approximately 90% to total metal uptake by the organelles. As cytosolic metal concentration increased, a greater accumulative role would be assumed for metal uptake by the organic anion exchanger so that at extreme metal concentrations, as might occur by food contamination, almost 90% of the metal removal would take place by anion exchange. Both exchangers contribute approximately 50% to metal uptake at concentrations around 75–100 μmol l^{-1}. Values for this figure were calculated from the kinetic constants displayed in Table 1.

kinetic constants from Table 1 to determine the percentage of total zinc uptake that would occur by way of each transport system over a proposed metal concentration range of 1 to 500 μmol l^{-1}. As shown in the figure, at low cytosolic zinc concentrations, as would occur during normal 'housekeeping' activities (i.e. below 25 μmol l^{-1} zinc), approximately 90% of zinc uptake would occur by the high affinity cation exchanger. However, during intervals of increased metal accumulation by the cell, a greater uptake of the metal into lysosomes takes place by the low affinity Zn-GSH/AKG conjugate exchanger. At the highest metal concentrations displayed (e.g. 500 μmol l^{-1}), almost 90% of the lysosomal metal transport would take place in conjunction with GSH (or AKG), while only 10–15% would enter the organelle by cation exchange. Because GSH (and/or AKG) concentration in the epithelial cytoplasm is upregulated by metal induction of metabolic events (Lash and Zalups, 1998), the low affinity Zn-GSH/AKG conjugate exchanger provides an ideal means by which to prevent cytoplasmic metal concentrations from exceeding physiological limits under most conditions that the cells may encounter.

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REFERENCES

Al-Mohanna, S. Y. and Nott, J. A. (1985). The accumulation of metals in the hepatopancreas of the shrimp, *Penaeus semisulcatus* de Haan (Crustacea. Decapoda) during the moult cycle. In *Marine Environmental Pollution* (ed. R. Halwagy, D. Clayton and M. Behbehani), pp. 195-209. Kuwait: Kuwait University Press.

Al-Mohanna, S. Y. and Nott, J. A. (1987). R-cells and the digestive cycle in *Penaeus semisulcatus* de Haan (Crustacea, Decapoda). *Mar. Biol.* **95**, 129-137.

Al-Mohanna, S. Y. and Nott, J. A. (1989). Functional cytology of the hepatopancreas of *Penaeus semisulcatus* (Crustacea, Decapoda) during the moult cycle. *Mar. Biol.* **101**, 535-544.

Beyersmann, D. and Haase, H. (2001). Functions of zinc in signaling, proliferation and differentiation of mammalian cells. *Biometals* **14**, 331-341.

Brouwer, M., Schlenk, D., Ringwood, A. H. and Brouwer-Hoexum, T. (1992). Metal-specific induction of metallothionein isoforms in the blue crab *Callinectes sapidus* in response to single- and mixed-metal exposure. *Arch. Biochem. Biophys.* **294**, 461-468.

Brouwer, M., Enghild, J., Hoexum-Brouwer, T., Thogersen, I. and Truncali, A. (1995). Primary structure and tissue-specific expression of blue crab (*Callinectes sapidus*) metallothionein isoforms. *Biochem. J.* **311**, 617-622.

Burlando, B., Viarengo, A., Pertica, M., Mancinelli, G., Marchi, B. and Orunesu, M. (1997). Effects of Hg^{2+} on Ca^{2+} dynamics in the scallop sarcoplasmic reticulum (*Pecten jacobaeus*). Protective role of glutathione. *Comp. Biochem. Physiol.* **116C**, 77-83.

Chavez-Crooker, P., Garrido, N. and Ahearn, G. A. (2002). Copper transport by lobster (*Homarus americanus*) hepatopancreatic mitochondria. *J. Exp. Biol.* **205**, 405-413.

Chavez-Crooker, P., Garrido, N., Pozo, P. and Ahearn, G. A. (2003). Copper transport by lobster (*Homarus americanus*) hepatopancreatic lysosomes. *Comp. Biochem. Physiol.* **135C**, 107-118.

Chou, H. F., Vadgama, J. and Jonas, A. J. (1992). Lysosomal transport of small molecules. *Biochem. Med. Metab. Biol.* **48**, 179-193.

Gibson, R. and Barker, P. L. (1979). The decapod hepatopancreas. *Oceanogr. Mar. Biol.* **17**, 285-346.

Hopfer, U., Nelson, K., Perrotto, J. and Isselbacher, K. J. (1973). Glucose transport in isolated brush border membrane from rat small intestine. *J. Biol. Chem.* **248**, 25-32.

Hopkin, S. P. (1989). *Ecophysiology Of Metals In Terrestrial Invertebrates*. London: Elsevier Applied Science.

Klasassen, C. D. (2001). *Toxicology*, 6th edn. New York: McGraw Hill.

Lash, L. H. and Zalups, R. K. (1998). Alterations in renal cellular glutathione metabolism after in vivo administration of a subtoxic dose of mercuric chloride. *J. Biochem. Toxicol.* **11**, 1-9.

Mandal, P. K., Mandal, A. and Ahearn, G. A. (2005). Physiological characterization of $^{45}Ca^{2+}$ and $^{65}Zn^{2+}$ transport by lobster hepatopancreatic endoplasmic reticulum. *J. Exp. Zool.* **303A**, 515-526.

Mandal, P. K., Mandal, A. and Ahearn, G. A. (2006). $^{65}Zn^{2+}$ transport by lobster hepatopancreatic lysosomal membrane vesicles. *J. Exp. Zool.* **305A**, 203-214.

Paquet, F., Germain, P. and Fritsch, P. (1993). Etude ultrastuctuale de la glande digestive du homard *Homarus gammarus*. Role des divers types cellulaires dans les processus digestifs. *Cah. Biol. Mar.* **35**, 15-37.

Pisoni, R. L. and Thoene, J. G. (1991). The transport systems of mammalian lysosomes. *Biochim. Biophys. Acta* **1071**, 351-373.

Segel, I. H. (1975). *Enzyme Kinetics*. New York: John Wiley and Sons.

Sokolova, I. M., Ringwood, A. H. and Johnson, C. (2005). Tissue-speciic accumulation of cadmium in subcellular compartments of eastern oysters *Crassostrea virginica* Gmelin (Bivalvia, Ostreidae). *Aquatic Toxicol.* **74**, 218-228.

Sterling, K. M., Mandal, P. K., Roggenbeck, B. A., Ahearn, S. E., Gerencser, G. A. and Ahearn, G. A. (2007). Heavy metal detoxification in crustacean epithelial lysosomes. role of anions in the compartmentalization process. *J. Exp. Biol.* **210**, 3484-3493.

Thamotharan, M., Lombardo, Y. B., Bawani, S. Z. and Adibi, S. A. (1997). An active mechanism for completion of the final stage of protein degradation in the liver, lysosomal transport of dipeptides. *J. Biol. Chem.* **272**, 11786-11790.

Van Weel, P. B. (1974). Hepatopancreas? *Comp. Biochem. Physiol.* **47A**, 1-9.

Verri, T., Mandal, A., Zilli, L., Bossa, D., Mandal, P. K., Ingrosso, L., Zonno, V., Vilella, S., Ahearn, G. A. and Storelli, C. (2001). D-glucose transport in decapod crustacean hepatopancreas. *Comp. Biochem. Physiol.* **130A**, 585-606.

Viarengo, A. (1994). Heavy metal cytotoxicity in marine organisms. Effects of Ca^{2+} homeostasis and possible alteration of signal transduction pathways. *Adv. Comp. Environ. Physiol.* **20**, 85-110.

Viarengo, A., Moore, M. N., Pertica, M., Mancinelli, G., Zanicchi, G. and Pipe, R. K. (1985a). Detoxification of copper in the cells of the digestive gland of mussels. The role of lysosomes and thioneins. *Sci. Total Environ.* **44**, 135-145.

Viarengo, A., Palmero, S., Zanicchi, G., Capelli, R., Vaissiere, R. and Orunesu, M. (1985b). Role of metallothioneins in Cu and Cd accumulation and elimination in the gill and digestive gland cells of *Mytilus galloprovincialis. Lam. Mar. Envir. Res.* **16**, 23-36.

Viarengo, A., Pertica, M., Canesi, L., Mazzucotelli, A., Orunesu, M. and Bouquegneau, J. M. (1989). Purification and biochemical characterization of a lysosomal copper-rich thionein-like protein involved in metal detoxification in the digestive gland of mussels. *Comp. Biochem. Physiol.* **93C**, 389-395.

Watanabe, T., Kiron, V. and Satoh, S. (1997). Trace minerals in fish nutrition. *Aquaculture* **151**, 185-207.

Zalups, R. and Barfuss, D. W. (2002). Renal organic anion transport system. A mechanism for the basolateral uptake of mercury-thiol conjugates along the pars recta of the proximal tubule. *Toxicol. Applied Pharmacol.* **182**, 234-243.