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Heavy metal detoxification in crustacean epithelial lysosomes: role of anions in the compartmentalization process

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Summary

Crustacean hepatopancreatic lysosomes are organelles of heavy metal sequestration and detoxification. Previous studies have shown that zinc uptake by lysosomal membrane vesicles (LMV) occurred by a vanadate- and thapsigargin-sensitive ATPase that was stimulated by a transmembrane proton gradient established by a co-localized V-ATPase associated with this organelle. In the present study, hepatopancreatic LMV from the American lobster *Homarus americanus* were prepared by standard centrifugation methods and $^{65}Zn^{2+}$, $^{36}Cl^{-}$, $^{35}SO_4^{2-}$ and ^{14}C-oxalate^{2-} were used to characterize the interactions between the metal and anions during vesicular detoxification events. Vesicles loaded with SO_4^{2-} or PO_4^{3-} led to a threefold greater steady-state accumulation of Zn^{2+} than similar vesicles loaded with mannitol, Cl^{-} or oxalate^{2-}. The stimulation of $^{65}Zn^{2+}$ uptake by intravesicular sulfate was SO_4^{2-} concentration dependent with a maximal enhancement at 500 μmol l^{-1}. Zinc uptake in the presence of ATP was proton-gradient enhanced and electrogenic, exhibiting an apparent exchange stoichiometry of $1Zn^{+}/3H^{+}$. $^{35}SO_4^{2-}$ and ^{14}C-oxalate^{2-} uptakes were both enhanced in vesicles loaded with intravesicular Cl^{-} compared to vesicles containing mannitol, suggesting the presence of anion countertransport. $^{35}SO_4^{2-}$ influx was a sigmoidal function of external [SO_4^{2-}] with 25 mmol l^{-1} internal [Cl^{-}], or with several intravesicular pH values (e.g. 7.0, 8.0 and 9.0). In all instances Hill coefficients of approximately 2.0 were obtained, suggesting that 2 sulfate ions exchange with single Cl^{-} or OH^{-} ions. $^{36}Cl^{-}$ influx was a sigmoidal function of external [Cl^{-}] with intravesicular pH of 7.0 and 9.0. A Hill coefficient of 2.0 was also obtained, suggesting the exchange of 2 Cl^{-} for 1 OH^{-}. ^{14}C-oxalate influx was a hyperbolic function of external [oxalate^{2-}] with 25 mmol l^{-1} internal [Cl^{-}], suggesting a 1:1 exchange of oxalate^{2-} for Cl^{-}. As a group, these experiments suggest the presence of an anion exchange mechanism exchanging monovalent for polyvalent anions. Polyvalent inorganic anions (SO_4^{2-} and PO_4^{3-}) are known to associate with metals inside vesicles and a detoxification model is presented that suggests how these anions may contribute to concretion formation through precipitation with metals at appropriate vesicular pH.

Key words: zinc transport, lysosomes, detoxification, sulfate, oxalate, chloride, anion exchange, heavy metals, lobster, *Homarus americanus*, V-ATPase, electrogenic transport.

Introduction

The crustacean hepatopancreas is a multifunctional organ with digestive and absorptive properties toward a variety of organic molecules and, in addition, is a site of heavy metal sequestration and detoxification. Detoxification of dietary metals by hepatopancreatic epithelial cells is a result of the simultaneous operation of several intracellular processes working in concert to maintain a low cytoplasmic concentration of these ions while extracellular concentrations may be highly variable (Ahearn et al., 2004a; Roesijadi and Robinson, 1994; Viarengo, 1989). Metallothioneins are low molecular mass proteins in cells that preferentially bind metals and are upregulated when external concentrations of metals rise (Al-Mohanna and Nott, 1985; Brouwer et al., 1989; Brouwer et al., 1992). Besides metallothioneins that reduce the concentrations of soluble cytoplasmic metals, mitochondria (Chavez-Crooker et al., 2002), endoplasmic reticulum (Mandal et al., 2005) and lysosomes (Mandal et al., 2006) are organelle centers of heavy metal sequestration that use membrane transport proteins to accumulate cytoplasmic cationic metals.

Previous studies with lobster (*Homarus americanus*) heptaopancreatic lysosomes have shown that both copper (Chavez-Crooker et al., 2003) and zinc (Mandal et al., 2006) are transported from hepatopancreatic epithelial cytoplasm into organelle interior by ATP-dependent carrier-mediated transporters that are sensitive to both vanadate and thapsigargin. In addition, both metals were cross-inhibited, suggesting the presence of a metal transporter with a relatively broad substrate specificity. Detoxification of heavy metals in organelles such as lysosomes requires the presence of a mechanism that sequesters the cation in a form that is relatively immobile and cannot be transferred back into the cytoplasm. Electron microprobe analyses of a variety of invertebrate epithelial cells indicate that metals are sequestered together with sulfate and phosphate in solid concretions within membrane-bound organelles (Al-Mohanna and Nott, 1985). While the composition of these

concretions has been evaluated in a wide variety of invertebrate species, the mechanisms whereby complexing anions are transferred into these sites of detoxification are unknown. This study examines the nature of anion transport by lobster hepatopancreatic lysosomal membranes and suggests a mechanism by which metal cations and polyvalent anions can be brought together to form detoxifying concretions in these cells.

Materials and methods

Animals

Live Atlantic lobsters *Homarus americanus* Milne-Edwards 1837, with a body mass of 500–700 g, were purchased from local commercial dealers in Jacksonville, FL, USA and maintained in a seawater holding tank at 15°C until needed for experimentation. Intermolt (molt stage estimated by gastrolith mass/carapace length ratio) lobsters were used for all experiments. Lobsters were provided with mussel meat for up to 15–20 days and all experiments were conducted only on animals that had been fasted for approximately 24 h to ensure an evacuated hepatopancreas.

Isolation of hepatopancreatic lysosomal membrane vesicles (LMV)

Hepatopancreatic lysosomal membrane vesicles (LMV) were prepared from fresh organs of individual lobsters. Hepatopancreatic tissue was quickly placed in chilled Buffer A (in mmol l^{-1}: 250 sucrose, 20 Hepes, 1 EDTA, 0.1 PMSF, adjusted to pH 7.0 with Tris base). The tissue was homogenized with a glass hand homogenizer and diluted tenfold in chilled Buffer A. The homogenate was centrifuged at 800 ***g*** for 10 min and the resulting supernatant was centrifuged at 20 000 ***g*** for 10 min. The pellet was re-suspended in Buffer B (in mmol l^{-1}: 250 mmol l^{-1} sucrose, 20 Hepes, adjusted to pH 7.0 with Tris base). The suspension was mixed with isotonic Percoll™ in the ratio of 9:11 (pellet suspended in Buffer B:isotonic Percoll™). The Percoll™ mixture was centrifuged at 40 000 ***g*** for 90 min. The brownish 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 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 at 18°C. An equal volume of ice-cold isotonic Percoll™ was added to the incubation mixture and centrifuged at 35 000 ***g*** for 30 min. The purified LMV (located on the top of the gradient as a brownish band) was re-suspended in Preloading Buffer (varied experiment to experiment).

An enzyme characterization of lobster hepatopancreatic LMV produced by the Percoll™ centrifugation method described above has been previously published (Mandal et al., 2006). In this study, the enrichments of three enzymes of known disparate cellular localization were used to show the purification of the LMV samples used in the present investigation. While the brush border enzyme marker, alkaline phosphatase, and the ER marker enzyme, NADPH-cytochrome *c* reductase, were not significantly enriched ($P>0.05$), the lysosomal enzyme, acid phosphatase, was purified by more than a factor of 12 in the final vesicle suspension compared to the original tissue homogenate. These data suggest that the LMV fractions used in this previous investigation, and also in the present study, were relatively pure lysosomal membranes and had minimal membrane contamination from other parts of the cell such as the plasma membrane or the endoplasmic reticulum.

$^{65}Zn^{2+}$, $^{36}Cl^{-}$, $^{35}SO_4^{2-}$ and ^{14}C-oxalate^{2-} transport measurements

Characteristics of $^{65}Zn^{2+}$ transport by isolated vesicles from hepatopancreatic lysosomal membranes were studied at room temperature (23°C). Experiments were initiated by diluting a small volume of vesicle suspension into a medium containing trace amounts of $^{65}ZnCl_2$ (+ unlabelled zinc sulfate), $K^{36}Cl^{-}$ (+ unlabelled KCl), $K_2{}^{35}SO_4^{2-}$ (+ unlabelled $K_2SO_4^{2-}$) or ^{14}C-oxalic acid (+ unlabelled oxalic acid). The composition of the final vesicle suspension solutions (inside vesicles) and incubation media (outside vesicles) are described separately for each experiment. Uptake of the radiolabelled substrate was initiated by rapidly mixing 20 ml of membrane suspension (150 mg of protein), preloaded with buffer (pH 7.0), with 180 ml of transport medium (described separately for each experiment) and incubating for appropriate time periods. Transport was terminated by addition of 2 ml (tenfold dilution) ice-cold buffer (stop solution) and the suspension was immediately collected under vacuum on a Millipore™ filter (HAWP, Billerica, MA, USA; 45 mm pore size), utilizing the Millipore™ filtration technique developed by Hopfer et al. (Hopfer et al., 1973). Filters were then dissolved in liquid scintillation cocktail (Ecolume™) and the radioactivity counted in a Beckman Coulter LS 6500 multi-purpose scintillation counter. Isotope uptake was expressed as pmol mg^{-1} protein s^{-1} or as nmol mg^{-1} protein s^{-1}. The protein content of the vesicle suspension was determined according to the Bradford proceedure (BioRad, Hercules, CA, USA), using bovine serum albumin as a standard. Displayed zinc activities were achieved using appropriate concentrations of zinc, NTA (nitriloacetic acid; *N*,*N*-bis[carboxymethyl]glycine), and ATP (0.2 mmol l^{-1}), using Winmax Chelator 2.0 software (Bers et al., 1994).

Isotope uptake into lysosomal vesicles was corrected for non-specific isotope binding (bound activity to exterior of vesicles and not transported to the vesicular interior) by injecting a sample of lysosomal vesicles and isotope directly into ice-cold stop solution without prior mixing. The resulting lysosomal vesicle suspension was then filtered, rinsed and counted as described previously. Resulting values for non-specific isotope binding were subtracted from total isotope uptake in each experiment, providing an index of transmembrane transport of the respective radiolabelled cation or anion. Time points are presented as means of 3–5 replicates and their associated standard errors (s.e.m.). Experiments were repeated at least twice with different animals. Statistical comparisons were made using Student's *t*-test where a value of $P<0.05$ was considered significant. Curve-fitting procedures were accomplished using Sigma Plot 9.0 software (Jandel, San Rafael, CA, USA), which provided an iterative best fit to experimental values.

Chemicals

$^{65}ZnCl_2$ was purchased from Oak Ridge National Laboratory, Oak Ridge, TN, USA and $^{36}Cl^{-}$, $^{35}SO_4^{2-}$ and ^{14}C-

oxalic acid were obtained from DuPont New England Nuclear Corp., Boston, MA, USA. Valinomycin, Zinc-sulfate, Tris, D-mannitol, and other reagent grade chemicals were purchased from Sigma Chemicals (St Louis, MO, USA), Fisher (Pittsburgh, PA, USA), or Bio-Rad (Hercules, CA, USA).

Results

Effect of intravesicular anions on $^{65}Zn^{2+}$ uptake

In order to see if the nature of intravesicular anions affected the uptake and equilibrium of $^{65}Zn^{2+}$ by hepatopancreatic lysosomal vesicles, vesicle preparations were filled with a selection of monovalent and polyvalent anions or mannitol and the rate of $^{65}Zn^{2+}$ uptake and final metal equilibrium level achieved in these vesicles were compared. As displayed in Fig. 1A, vesicles preloaded with mannitol at pH 7.0 exhibited a slow uptake rate of 25 μmol l^{-1} $^{65}Zn^{2+}$ and an equilibrium attained at 30 s of about 2 pmol mg^{-1} protein. In contrast, when vesicles were preloaded with either 25 μmol l^{-1} SO_4^{2-} or 25 μmol l^{-1} PO_4^{3-} at pH 7.0, the uptake rate of 25 μmol l^{-1} $^{65}Zn^{2+}$ was twice as fast as in mannitol-loaded vesicles and the 20–30 s equilibrium for the metal was also twice that of the vesicles without preloaded anions. There was no significant difference (P>0.05) between the rates of metal uptake or the eventual metal equilibrium attained in vesicles preloaded with the two polyvalent anions.

As shown in Fig. 1B, preloading lysosomal vesicles with either the monovalent anion, Cl^- (25 μmol l^{-1}) at pH 7.0, or divalent organic anion, oxalate^{2-} (25 μmol l^{-1}) at pH 7.0, resulted in 25 μmol l^{-1} $^{65}Zn^{2+}$ uptake rates and metal equilibrium values that were not significantly different (P>0.05) than those achieved by preloading the vesicles with mannitol. These results suggest that the inorganic polyvalent anions, SO_4^{2-} and PO_4^{3-}, at pH 7.0 were able to form an intravesicular association with $^{65}Zn^{2+}$ that allowed twice as much metal to accumulate within these compartments than occurred when mannitol, inorganic monovalent anions (Cl^-) or organic polyvalent anions (oxalate^{2-}) were present.

Because 25 μmol l^{-1} SO_4^{2-} was one of the polyvalent inorganic anions that led to enhanced vesicle accumulation of 25 μmol l^{-1} $^{65}Zn^{2+}$, an experiment was conducted to see what effect a wide range of intravesicular SO_4^{2-} concentrations would have on the metal uptake by these membrane preparations. Fig. 2 shows that increasing intravesicular SO_4^{2-} from 25 to 1000 μmol l^{-1} resulted in a stepwise increase in uptake rate and equilibrium of 25 μmol l^{-1} $^{65}Zn^{2+}$ by hepatopancreatic lysosomal vesicles. Metal uptakes at 500 and 1000 μmol l^{-1} SO_4^{2-} were not significantly different (P>0.05) from one another, but were significantly different (P<0.01) from those at lower SO_4^{2-} concentrations, suggesting that the mechanism resulting in the enhancement of metal uptake reached a maximum around an intravesicular SO_4^{2-} concentration of approximately 500 μmol l^{-1}.

$^{65}Zn^{2+}$ uptake by lysosomal vesicles is ATP-dependent and electrogenic

Previous studies examining the nature of $^{65}Zn^{2+}$ transport by hepatopancreatic lysosomal membrane vesicles showed that metal uptake by these vesicles was stimulated by the presence

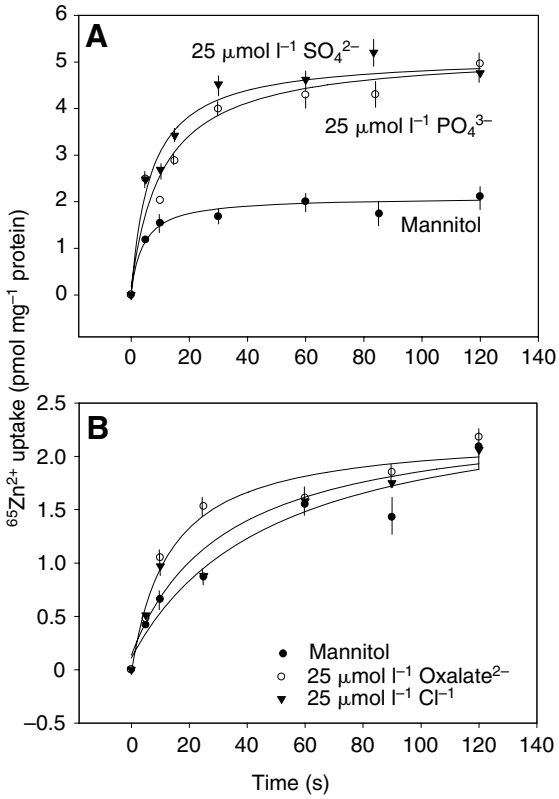

Fig. 1. (A) Effects of intravesicular polyvalent inorganic anions and mannitol on the time course of 25 μmol l^{-1} $^{65}Zn^{2+}$ uptake by hepatopancreatic lysosomal membrane vesicles (LMV). Vesicles were loaded with 200 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris, pH 7.0, and 25 μmol l^{-1} K_2SO_4, K_3PO_4 or mannitol, and were then incubated in a medium containing 200 mmol l^{-1} mannitol, 25 μmol l^{-1} ^{65}Zinc-sulfate, 0.5 mmol l^{-1} NTA, 0.2 mmol l^{-1} ATP and 20 mmol l^{-1} Hepes/Tris, pH 7.0. (B) Effects of intravesicular monovalent inorganic anions (Cl^{-1}), polyvalent organic anions (oxalate^{2-}) and mannitol at pH 7.0 on the time course of 25 μmol l^{-1} $^{65}Zn^{2+}$ uptake by LMV. Uptake conditions as in A except that in this instance vesicles were loaded with 25 μmol l^{-1} oxalic acid, NaCl or mannitol. Experiments were conducted in triplicate; values are means ± 1 s.e.m. at each time point.

of ATP in the incubation medium and this nucleotide stimulation was inhibited by vanadate and thapsigargin (Mandal et al., 2006). In addition, $^{65}Zn^{2+}$ uptake was also stimulated by an acidic vesicle interior (pH_i=5.0; pH_o=7.0). These results suggested that zinc transport occurred by a Zn^{2+}/H^+ antiport ATPase, but the exchange stoichiometry of the transporter was unclear. In order to clarify the stoichiometric nature of this exchanger an experiment was conducted using valinomycin (potassium ionophore) and different concentrations of K^+ across the vesicle membrane. Under these conditions, a transmembrane potential was generated from one side of the membrane to the other. If the Zn^{2+}/H^+ antiporter was electroneutral (e.g. 1 $Zn^{2+}/2H^+$) a transmembrane electrical potential difference would not influence the rate of this exchange, while an electrogenic exchange (e.g. 1 $Zn^{2+}/1H^+$ or 1 $Zn^{2+}/3H^+$) would be significantly affected by the imposition of a transmembrane potential.

The influx of $^{65}Zn^{2+}$ was stimulated by a decrease in

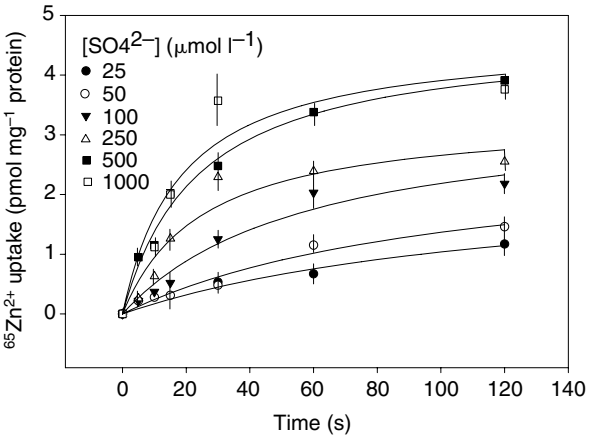

Fig. 2. Effect of varying intravesicular SO_4^{2-} concentration on the time course of 25 μmol l^{-1} $^{65}Zn^{2+}$ uptake by LMV. Vesicles were loaded with 200 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris, pH 7.0 and K_2SO_4 concentrations of 25, 50, 100, 250, 500 or 1000 μmol l^{-1}, and were then incubated in a medium containing 200 mmol l^{-1} mannitol, 25 μmol l^{-1} ^{65}Zinc-sulfate, 0.5 mmol l^{-1} NTA, 0.2 mmol l^{-1} ATP, and 20 mmol l^{-1} Hepes/Tris, pH 7.0. Experiments were conducted in triplicate; values are means ± 1 s.e.m. at each time point.

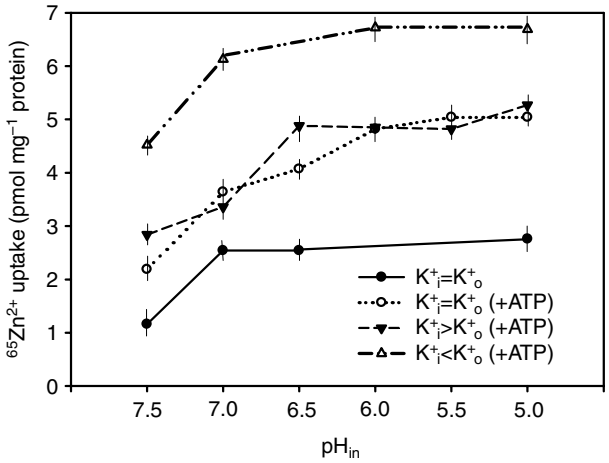

Fig. 3. Effects of intravesicular pH (pH 7.5–5.0), extravesicular ATP (1 mmol l^{-1}), and induced membrane potential on the influx (10 s uptake) of 25 μmol l^{-1} $^{65}Zn^{2+}$. A transmembrane electrical potential was induced by equilibrating the vesicles with 50 μmol l^{-1} valinomycin, varying the extravesicular K^+ concentration [0 mmol l^{-1} (K_i>K_o), 100 mmol l^{-1} (K_i=K_o) or 200 mmol l^{-1} (K_i<K_o)], and maintaining a constant intravesicular K^+ concentration (100 mmol l^{-1}). Vesicles were loaded with 100 mmol l^{-1} K_2SO_4, either 20 mmol l^{-1} Hepes/Tris (pH 6.5–7.5) or Mes/Tris (pH 5.0–6.0), 50 μmol l^{-1} valinomycin, and appropriate mannitol to maintain osmolarity. Loaded vesicles were then incubated in media containing 25 μmol l^{-1} ^{65}Zinc-sulfate, 0, 100 or 200 mmol l^{-1} K_2SO_4, 0.2 or 1.0 mmol l^{-1} ATP (+ATP), 0.5 mmol l^{-1} NTA, 20 mmol l^{-1} Hepes/Tris, pH 7.0, and mannitol to maintain osmolarity. The experiment was conducted in triplicate; values are means ± 1 s.e.m. at each time point.

intravesicular pH and by the presence of external ATP (Fig. 3). Furthermore, in the presence of ATP, metal transport was enhanced by an intravesicular positive electrical potential difference (e.g. K^+_i<K^+_o) compared to when the vesicles were either short-circuited (e.g. K^+_i=K^+_o) or contained an intravesicular negative electrical potential difference (e.g. K^+_i>K^+_o). These results suggest that because an electrically positive intravesicular potential difference increased the uptake of $^{65}Zn^{2+}$, more positive charge must have been transferred out of the vesicle than into the vesicle during the exchange of zinc and protons. The minimum transport stoichiometry that could account for this exchange would be $1Zn^{2+}/3H^+$ and this proposed ratio is consistent with the data displayed in Fig. 3.

Countertransport of anions by lysosomal vesicles

Because intravesicular polyvalent inorganic anions like SO_4^{2-} and PO_4^{3-} enhanced the equilibrium accumulation of $^{65}Zn^{2+}$ within hepatopancreatic lysosomal vesicles, the nature of the uptake process facilitating the transfer of these anions from the cytoplasm was investigated. Two groups of vesicles were prepared. One group was loaded with mannitol and Hepes/Tris at pH 7.0 only and the other had mannitol and intravesicular chloride at pH 7.0. These two vesicle groups were then incubated in media containing $^{35}SO_4^{2-}$ or ^{14}C-oxalate^{2-} and the time course of isotope uptake into both membrane groups was followed. Fig. 4A shows that $^{35}SO_4^{2-}$ uptake was stimulated in vesicles containing intravesicular Cl^- compared to that shown by mannitol-loaded vesicles. Fig. 4B shows a similar response to intravesicular Cl^- by ^{14}C-oxalate^{2-} uptake. These results suggest the presence of an anion countertransport process in hepatopancreatic LMV, but do not clarify any of its properties except that it exchanges anions.

Polyvalent anion exchange kinetics with intravesicular chloride

In order to more fully describe the features of LMV anion exchange, additional experiments were conducted describing the characteristics of anion influx kinetics into Cl^--loaded vesicles. Fig. 5A shows $^{35}SO_4^{2-}$ influx kinetics into lysosomal vesicles preloaded with 25 mmol l^{-1} Cl^-. As displayed in this figure, the influx of the divalent anion was a sigmoidal function of its cytoplasmic concentration when 25 mmol l^{-1} intravesicular Cl^- was present inside the preparation. Under these conditions, $^{35}SO_4^{2-}$ influx followed the Hill equation of multi-site cooperativity shown below:

$$J_{SO_4} = J_{max}\,[SO_4]^n / K_m{}^n + [SO_4]^n , \quad (1)$$

where J_{SO_4} is sulfate^{2-} influx at any given [SO_4^{2-}], J_{max} is apparent maximum sulfate^{2-} influx rate (mmol mg^{-1} protein s^{-1}), $K_m{}^n$ (mmol l^{-1}) is apparent binding affinity of the transporter for the anion corrected for multi-site cooperative interactions, $[SO_4^{2-}]^n$ is sulfate^{2-} concentration with multi-site corrections (mmol l^{-1}), and n is the apparent Hill coefficient, which is an estimate of the number of SO_4^{2-} ions transported during each transport cycle. The kinetic constants for J_{SO_4} were $K_m{}^n$=9.09±0.64 mmol l^{-1}, J_{max}=342±18 nmol mg^{-1} protein s^{-1}, and n=2.71±0.51 (Fig. 5A). The results of this experiment suggest the presence of an anion exchange process involved in the simultaneous

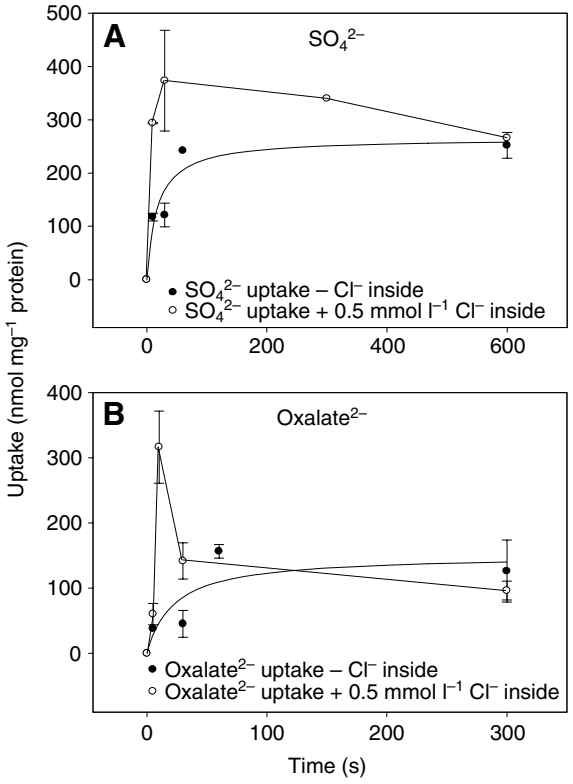

Fig. 4. Effect of preloaded Cl^- on the time course of $^{35}SO_4^{2-}$ and ^{14}C-oxalate^{2-} uptake by hepatopancreatic lysosomal membrane vesicles. (A) Vesicles were loaded with 200 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris, pH 7.0, with or without 0.5 mmol l^{-1} KCl, and were incubated in media containing 200 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris, pH 7.0, with or without 0.5 mmol l^{-1} potassium gluconate and 5 mmol l^{-1} $K_2{}^{35}SO_4$. (B) Vesicles were loaded with 200 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris, pH 7.0, with or without 0.5 mmol l^{-1} KCl, and were incubated in media containing 200 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris, pH 7.0, with or without 0.5 mmol l^{-1} potassium gluconate, and 5 mmol l^{-1} ^{14}C-oxalate^{2-}. Experiments were in triplicate; values are means ± 1 s.e.m. at each time point.

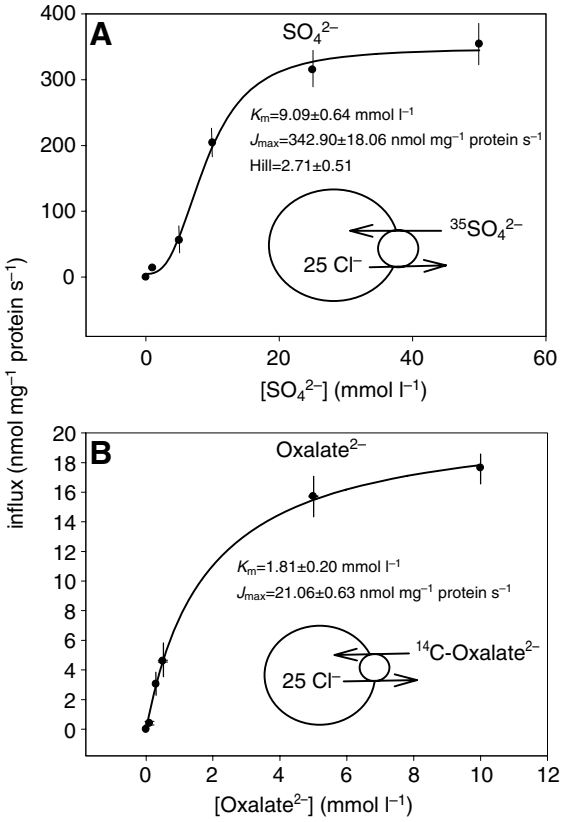

Fig. 5. Influx kinetics (10 s uptake) of polyvalent inorganic ($^{35}SO_4^{2-}$) and organic (^{14}C-oxalate^{2-}) anions into Cl^--loaded (25 mmol l^{-1} Cl^-) LMV. (A) Vesicles were loaded with 200 mmol l^{-1} mannitol, 25 mmol l^{-1} KCl, 20 mmol l^{-1} Hepes/Tris, pH 7.0 and were incubated in media containing 200 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris, pH 7.0, and $K_2{}^{35}SO_4$ concentrations from 1 to 50 mmol l^{-1}. (B) Vesicles were loaded with 200 mmol l^{-1} mannitol, 25 mmol l^{-1} KCl, 20 mmol l^{-1} Hepes/Tris, pH 7.0 and incubated in media containing 200 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris, pH 7.0 and ^{14}C-oxalic acid concentrations from 0.1 to 10 mmol l^{-1}. Experiments were in triplicate; values are means ± 1 s.e.m. Lines drawn through the curves were computed using Sigma Plot 10.0 Software (Jandal).

transfer of monovalent and divalent anions across the lysosomal membrane. It is unclear whether this exchange process is electrogenic or electroneutral, but in either instance it results in the uptake of sulfate from the cytoplasm.

Fig. 5B displays a similar exchange process between the organic divalent anion, ^{14}C-oxalate^{2-}, and intravesicular Cl^-. In contrast with the results disclosed for sulfate^{2-} (Fig. 5A), influx kinetics for oxalate^{2-} followed the hyperbolic Michaelis–Menten relationship described below:

$$J_{Ox} = J_{max}[Ox^{2-}] / K_m+[Ox^{2-}] , \quad (2)$$

where J_{Ox} is oxalate influx at any given [oxalate^{2-}], K_m is the apparent binding affinity of the transporter for the divalent anion, J_{max} is apparent maximal oxalate^{2-} influx rate (nmol mg^{-1} protein s^{-1}), and [Ox^{2-}] is the oxalate^{2-} concentration (mmol l^{-1}). The kinetic constants for J_{Ox} were K_m=1.81±0.20 mmol l^{-1} and J_{max}=21.1±0.6 nmol mg^{-1} protein s^{-1}. As with sulfate^{2-} influx, these data suggest that ^{14}C-oxalate^{2-} was transported into these vesicles in exchange for intravesicular Cl^-, but the stoichiometric nature of this exchange is unclear. It is likely that the same antiporter was responsible for exchanging both sulfate^{2-} and oxalate^{2-} for Cl^-.

Lysosomal $^{36}Cl^-$ uptake occurs by exchange with OH^-

Because Cl^- ion acted as a 'common currency' for exchange with different divalent anions (e.g. Fig. 5), the question arose as to how this monovalent anion was taken up by lysosomal vesicles. Fig. 6 displays the results of an experiment examining the nature of $^{36}Cl^-$ influx into lysosomal membrane vesicles that were loaded at pH 7.0 or 9.0 (external pH 7.0 at both times), thereby providing only hydroxyl ions as a potential anion substrate for exchange with external $^{36}Cl^-$. As indicated in this figure, Cl^- influx was a sigmoidal function of external chloride concentration at each intravesicular pH condition and followed the Hill equation for multisite cooperativity between binding

ligands as described previously for $^{35}SO_4^{2-}$ influx into these vesicles:

$$J_{Cl} = J_{max}\,[Cl^-]^n / K_m{}^n + [Cl^-]^n , \quad (3)$$

where J_{Cl} is Cl^- influx (nmol mg^{-1} protein s^{-1}), J_{max} is maximal Cl^- influx (nmol mg^{-1} protein s^{-1}), $K_m{}^n$ is an apparent binding affinity constant (mmol l^{-1}), $[Cl^-]^n$ is the external Cl^- concentration (mmol l^{-1}) and n is the Hill coefficient.

The data reported in this figure indicate that there were no significant differences (P>0.05) between the apparent binding affinities (e.g. $K_m{}^n$) or the relative Hill coefficients (n) at the two pH conditions, but there was a significant (P<0.01) increase in J_{max} as the internal pH was raised from pH 7.0 to 9.0. In addition, the sigmoidal nature of the influx curves and a Hill coefficient of approximately 2.0 in each instance, suggested that two Cl^- ions were transported across the vesicular membrane during each transport event. Because OH^- ions were the only anion substrate within the vesicles at each of these conditions, the data indicate that these membranes possess a monovalent anion exchanger that exhibited either an antiport stoichiometry of $2Cl^-/1OH^-$ or $2Cl^-/2OH^-$.

In order to clarify the transport exchange stoichiometry between Cl^- ions and OH^- ions during $^{36}Cl^-$ influx into lysosomal membrane vesicles, $^{36}Cl^-$ influx was measured at four different extravesicular [Cl^-] (2.5, 5, 15 and 35 mmol l^{-1} Cl^-) over a range of different intravesicular pH values and the $^{36}Cl^-$ influx values are expressed as a function of intravesicular [OH^-] (nmol l^{-1}). As the data presented in Fig. 7 indicate, $^{36}Cl^-$ influx was a hyperbolic function of intravesicular [OH^-] at each extravesicular [Cl^-] from 2.5 to 35 mmol l^{-1} Cl^-. These results suggest that $^{36}Cl^-/OH^-$ exchange followed a modified Michaelis–Menten equation for carrier-mediated transport as shown below:

$$J_{Cl} = J_{max}\,[OH^-] / K_m + [OH^-] , \quad (4)$$

where in this instance J_{Cl} is Cl^- influx by exchange with intravesicular OH^- ions (nmol mg^{-1} protein s^{-1}), J_{max} is maximal Cl^- influx (nmol mg^{-1} protein s^{-1}), K_m is an apparent binding affinity constant of the exchanger for OH^- ions (nmol l^{-1}), and [OH^-] is the intravesicular concentration of hydroxyl ions (nmol l^{-1}).

While small changes in apparent Cl^- influx K_m ([OH^-] resulting in one-half maximal $^{36}Cl^-$) occurred over the range of Cl^- concentrations used, a 12.5-fold increase in Cl^- influx J_{max} took place from 2.5 to 35 mmol l^{-1} Cl^{-1}. These results implied a far greater effect on maximal $^{36}Cl^-$ influx rate of intravesicular [OH^-] than on the apparent binding of hydroxyl ions to the exchanger (e.g. K_m). Because the $^{36}Cl^-$ influx K_m values in all instances were in the acidic pH range, it can be argued that under normal physiological conditions where lysosomes are acidic, the anion antiporter would be saturated with intravesicular OH^- ions and anion exchange would be rate-limited by the cytoplasmic concentration of exchangeable anion substrates. Furthermore, the hyperbolic nature of the Cl^- influx curve over the [OH^-] used implies an electrogenic exchange transport stoichiometry of $2Cl^-/1OH^-$.

Lysosomal $^{35}SO_4^{2-}$ uptake occurs by exchange with OH^-

A series of experiments examining the nature of $^{35}SO_4^{2-}$ influx into lysosomal vesicles was performed following the protocol outlined for the investigation of $^{36}Cl^-$ influx into these preparations as described in Figs 6 and 7. In this instance vesicles were loaded at pH 7.0, 8.0 and 9.0 and were incubated in media containing $^{35}SO_4^{2-}$ at concentrations from 2.5 to 50 mmol l^{-1}. As in the previous series of experiments, OH^- ions were the only exchangeable anion substrate inside the vesicles. Sigmoidal influx kinetics, as described in Eqn 1, were obtained for SO_4^{2-} influx as a function of external [SO_4^{2-}] at each of the chosen internal pH conditions (Fig. 8). Results indicated that both sulfate^{2-} influx $K_m{}^n$ and J_{max} increased significantly (P<0.01) from pH 7.0 to 9.0, but a greater change was observed in the J_{max} parameters than in the apparent affinity values. A small increase (P<0.02) was also observed in the magnitude of the Hill coefficient n over the pH range examined, but all values approximated 2.0, as was found for Cl^- influx under similar conditions (Fig. 6).

To clarify the exchange stoichiometry between $^{35}SO_4^{2-}$ influx into and OH^- efflux from lysosomal vesicles, an experiment was conducted similar to that described in Fig. 7 for $^{36}Cl^-/OH^-$ exchange. As before, $^{35}SO_4^{2-}$ influx was measured into vesicles loaded at several internal pH values and were incubated in media containing either 5 or 10 mmol l^{-1} $^{35}SO_4^{2-}$. $^{35}SO_4^{2-}$ influx is expressed as a function of internal [OH^-] (nmol l^{-1}) in Fig. 9. SO_4^{2-} influx was a hyperbolic function of intravesicular [OH^-]

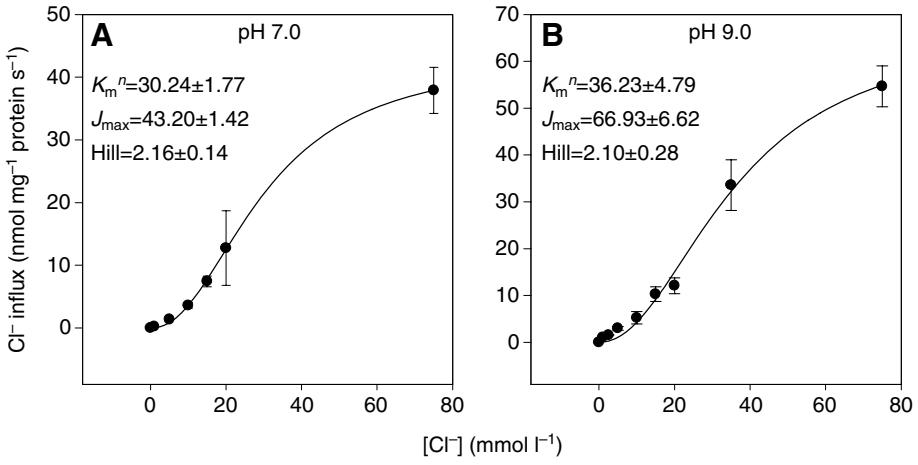

Fig. 6. Effect of intravesicular pH on $^{36}Cl^-$ influx kinetics in hepatopancreatic LMV. In both instances extravesicular pH was held at 7.0. Vesicles were loaded with 200 mmol l^{-1} mannitol and 20 mmol l^{-1} Hepes/Tris, pH 7.0 (A) or pH 9.0 (B). Loaded vesicles were then incubated in media containing 1, 5, 10, 15, 20 or 75 mmol l^{-1} $K^{36}Cl^-$, 20 mmol l^{-1} Hepes/Tris, pH 7.0, and mannitol to maintain osmolarity. The experiment was repeated in triplicate; values are means ± 1 s.e.m. The sigmoidal curves were drawn through the data using Sigma Plot software (Jandal).

and followed the modified Michaelis–Menten equation (Eqn 4) above except that the respective kinetic parameters related to SO_4^{2-}, rather than Cl^-, transfer. In this instance increasing external [SO_4^{2-}] from 5 to 10 mmol l^{-1} resulted in apparent K_m values for OH^- binding that were not significantly different (P>0.05). In contrast, SO_4^{2-} influx J_{max} values were significantly (P<0.01) greater at 10 mmol l^{-1} SO_4^{2-}. The hyperbolic nature of the influx curve in this experiment suggests an electrogenic exchange stoichiometry between the polyvalent and monovalent anions of 2 SO_4^{2-}/1 OH^-.

Discussion

Lysosomes are multi-functional intracellular organelles. It is widely accepted that a major role of these organelles in a wide variety of cell types is the disassembly of macromolecules through the use of enzymatic digestion in an acidic microenvironment (Chou et al., 1992; Pisoni and Thoene, 1991). In some cells such as renal proximal tubular cells, filtered plasma proteins are reabsorbed by endocytosis and are transferred to lysosomes for degradation (Gekle, 1998). This breakdown is taken to tri- and dipeptides, which are exported from lysosomes to cytoplasm where final hydrolysis to amino acids occurs using a lysosomal membrane oligopeptide transporter (Zhou et al., 2000). Similar hydrolysis of other macromolecules such as nucleotides, sugars and protein-associated vitamins (e.g. vitamin B_{12}) occurs in these organelles with monomeric subunits being transferred to the cytoplasm using a variety of membrane-bound lysosomal transport proteins (Pisoni and Thoene, 1991).

In addition to its macromolecular degradation role, lysosomal membranes possess a number of ion transport proteins for transferring both cations and anions between the intralysosomal compartment and the cytoplasm (Pisoni and Thoene, 1991; Chou et al., 1992; Dell'Angelica et al., 2000). Both proton and calcium ATPases have been described for lysosomal membranes (Pisoni and Thoene, 1991; Chou et al., 1992). The V-ATPase was considered responsible for lysosomal acidification, while the Ca-ATPase may help regulate cytoplasmic calcium activity. Calcium transport by human lysosomal membranes was inhibited by other divalent cations (Cd^{2+}>Hg^{2+}>Zn^{2+}>Mg^{2+}>Ba^{2+}>Sr^{2+}), but appeared insensitive to monovalent and trivalent cations (Lemons and Thoene, 1991). Sulfate^{2-}, phosphate^{3-}, molybdate^{4-} and Cl^- are transported across vertebrate lysosomal membranes (Pisoni and Thoene, 1991; Chou et al., 1992). These transport systems are affected by pH and the presence of other anions on the trans-side of a membrane preparation. Jonas and Jobe (Jonas and Jobe, 1990) described sulfate^{2-} transport across rat liver lysosomal membrane in exchange for Cl^- and suggest the presence of an anion exchanger in this organelle that is regulated by pH or membrane potential. Phosphate^{3-} transport in human fibroblast lysosomes is strongly affected by pH, but appears highly specific as certain other anions (e.g. SO_4^{2-}, HCO_3^-, Cl^- or DIDS) have no effect on its transfer (Pisoni and Thoene, 1991). However, arsenate^{3-} was a strong competitive inhibitor of phosphate^{3-} transport in this system with both substrate and inhibitor having similar binding constants (phosphate^{2-} K_m=5 μmol l^{-1}; arsenate^{2-} K_i=7 μmol l^{-1}). None of the studies that characterized anion transport by lysosomal membranes considered the interactions within the organelle that may result from the simultaneous transport of divalent ions from the cytosol.

Another function of lysosomes is the sequestration of heavy metals such as zinc and copper. Vertebrate lysosomes are known to store zinc by transporting the metal from cytosol to organelle interior by a Znt2 transport protein (McMahon and Cousins, 1998; Liuzzi and Cousins, 2004). In the case of copper, this metal is accumulated in lysosomes as a result of the activities of copper ATPases (ATP7A and ATP7B) or

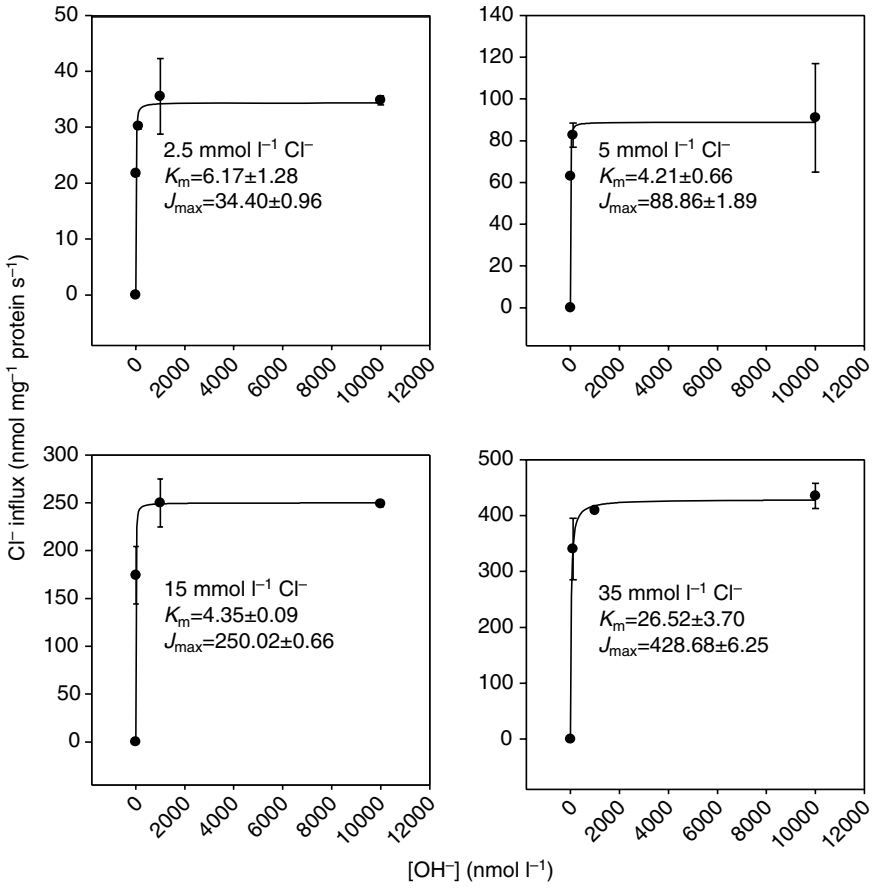

Fig. 7. Effect of intravesicular OH^- concentration on $^{36}Cl^-$ influx (2.5, 5, 15, and 35 mmol l^{-1} Cl^-; 5 s uptakes) in hepatopancreatic LMV. In all instances the extravesicular pH was held at 7.0. Vesicles were loaded with 200 mmol l^{-1} mannitol, and either 20 mmol l^{-1} Hepes/Tris or Mes/Tris, pH 6.0, 7.0, 8.0 and 9.0. Loaded vesicles were then incubated in media containing 2.5, 5, 15 and 35 mmol l^{-1} $K^{36}Cl^-$, 20 mmol l^{-1} Hepes/Tris, pH 7.0, and mannitol to maintain osmolarity. The experiment was repeated in triplicate; values are means ± 1 s.e.m. The hyperbolic curves were drawn through the data using Sigma Plot software (Jandal).

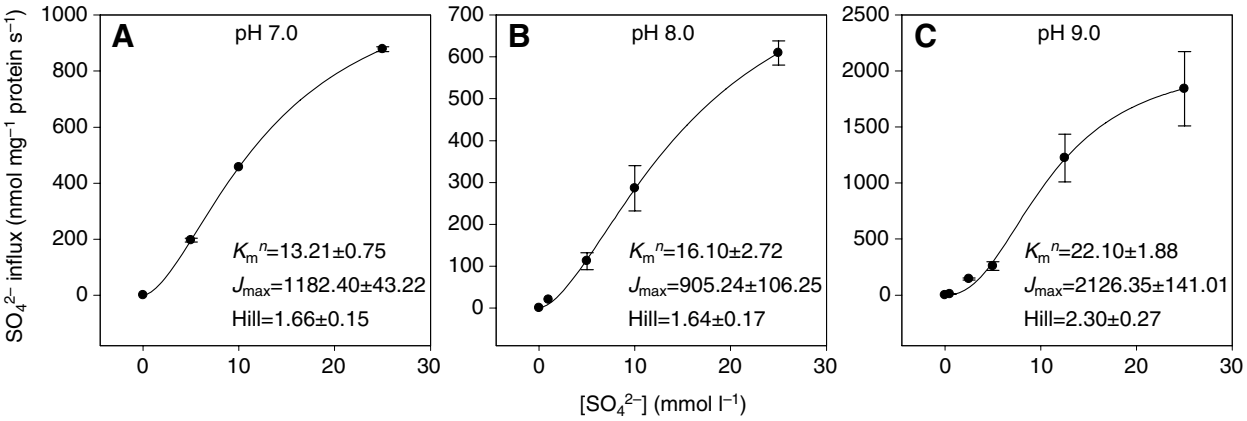

Fig. 8. Effect of intravesicular pH on $^{35}SO_4^{2-}$ influx kinetics in hepatopancreatic LMV. In all cases extravesicular pH was held at pH 7.0. Vesicles were loaded with 200 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris at pH 7.0 (A), 8.0 (B) and 9.0 (C). Loaded vesicles were then incubated in media containing 2.5, 5, 10, 25 and 50 mmol l^{-1} $K_2{}^{35}SO_4$, 20 mmol l^{-1} Hepes/Tris, pH 7.0, and mannitol to maintain osmolarity. The experiment was repeated in triplicate; values are means ± 1 s.e.m. The sigmoidal curves were drawn through the data using Sigma Plot software (Jandel).

facilitated diffusion systems of the Ctr2 or Ctr6 copper transporter isoforms (Bellemare et al., 2002).

In invertebrate cells, lysosomes are known to sequester a number of cations of both biological relevance (e.g. calcium, copper, zinc, iron) and non-relevance (e.g. cadmium, mercury, lead) in association with anions such as sulfate^{2-} and phosphate^{3-} in solid concretions that effectively remove them from cytoplasmic or plasma functionality. These concretions may serve as temporary storage facilities for cations involved in frequent physiological operations such as exoskeletal molting in crustaceans, where lysosomal calcium may be periodically mobilized or stored in cells of various tissues as needed to harden the newly synthesized, and still soft, exoskeletal components. Lysosomal storage of biologically important metals such as copper, zinc and iron may occur in similar endosomes side by side with calcium-containing organelles, but metal reclamation from the depots may occur at rates which are dictated by other physiological needs of the animal such as copper requirements in hemocyanin synthesis or zinc needs in efficient enzymatic activities. Lastly, some metals that have no known biological relevance, such as lead, cadmium and mercury, may be accumulated in certain lysosomes and retained there for the life of the cell, eventually being excreted from the animal through the gastrointestinal tract or some other evacuation site, effectively detoxifying the metals through long-term sequestration.

The experiments reported in this study extend those previously reported for zinc and copper transport by lobster hepatopancreatic lysosomes (Ahearn et al., 2004a; Ahearn et al., 2004b; Chavez-Crooker et al., 2003; Mandal et al., 2006) and for zinc transport by lobster hepatopancreatic endoplasmic reticulum (ER) (Mandal et al., 2005). In these previous studies membranes of both organelles were reported to possess ATP-dependent, thapsigargin- and vanadate-sensitive calcium and metal transport systems that were suggested to play a role in regulating cytosolic concentrations of these divalent cations. The suggestion was made that apparently different isoforms of the ER SERCA (sarco-endoplasmic reticulum calcium ATPase) may be localized at the membranes of both organelles and serve similar regulatory properties in the two sites (Ahearn et al., 2004b). However, the molecular identity of neither transporter was disclosed in these studies and only their relative pharmacological responses were noted.

Data presented in Figs 1 and 2 suggest that the polyvalent

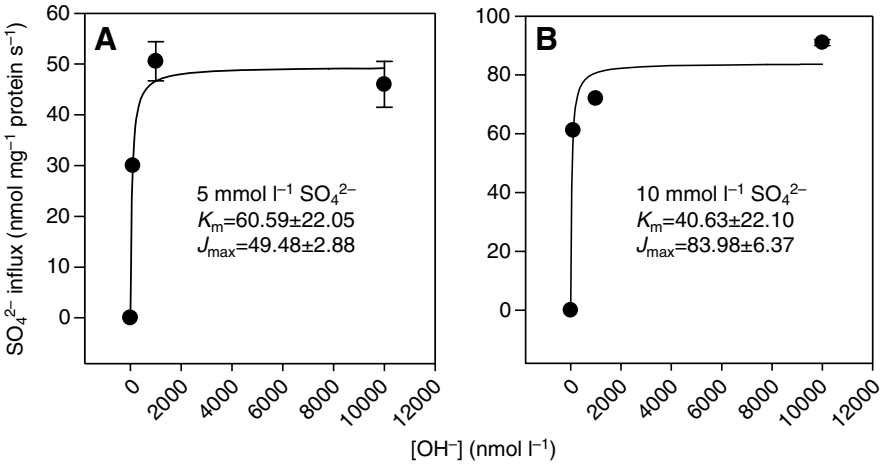

Fig. 9. Effect of intravesicular OH^- concentration on $^{35}SO_4^{2-}$ influx at 5 mmol l^{-1} (A) and 10 mmol l^{-1} SO_4^{2-} (B) (5 s uptakes) in hepatopancreatic LMV. In both cases the extravesicular pH was held at 7.0. Vesicles were loaded with 200 mmol l^{-1} mannitol and either 20 mmol l^{-1} Hepes/Tris or Mes/Tris, pH 7.0, 8.0 and 9.0. Loaded vesicles were then incubated in media containing either 5 or 10 mmol l^{-1} $K_2{}^{35}SO_4$, 20 mmol l^{-1} Hepes/Tris, pH 7.0 and mannitol to maintain osmolarity. The experiment was repeated in triplicate; values are means ± 1 s.e.m. The hyperbolic curves drawn through the data using Sigma Plot software (Jandal).

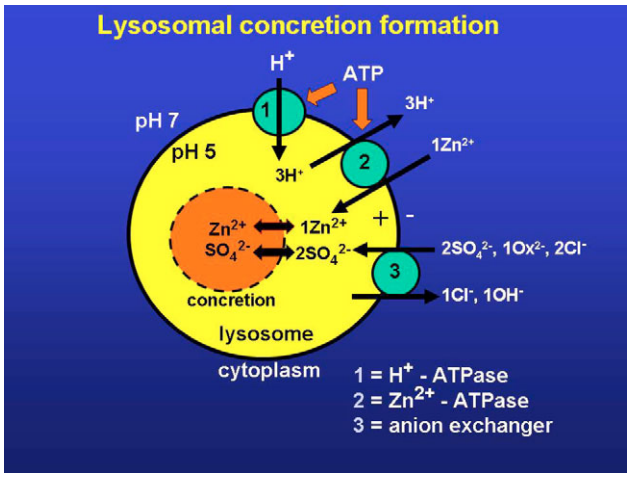

Fig. 10. Working model of the role of polyvalent anions in hepatopancreatic lysosomal heavy metal sequestration and detoxification. Membrane-bound, ATP-dependent, V-ATPase (Protein 1) transfers protons into the vesicle interior, creating a decrease in pH, an accumulation of hydrogen ions, and an inside-positive membrane potential. The outwardly directed proton gradient and positive vesicular interior provide the driving force for the asymmetric exchange of cytosolic divalent metals for intravesicular hydrogen ions by an ATP-dependent Zn^{2+}-ATPase, or a $3H^+/1Zn^{2+}$ exchanger (Protein 2). Polyvalent cytosolic anions such as sulfate^{2-} or phosphate^{3-} exchange with intravesicular monovalent anions such as Cl^- or OH^- by a second asymmetric antiporter (Protein 3), which uses the membrane potential as a driving force for exchange. Both divalent metals and polyvalent anions increase in concentration inside vesicles at acidic pH and are retained because they cannot be accommodated on the intravesicular binding sites of the exchangers. Divalent metals and polyvalent anions form precipitates (concretions) as the V-ATPase decreases in activity and the intravesicular pH rises.

anions SO_4^{2-} and PO_4^{3-} were more effective in metal accumulation within hepatopancreatic lysosomes than were uncharged solutes (mannitol), monovalent anions (Cl^-), or divalent organic cations (oxalate^{2-}). In addition, Fig. 3 provides, for the first time, a proposed exchange stoichiometry of the zinc transporter in these membranes. As a result of the action of an ATP-dependent V-ATPase (Chavez-Crooker et al., 2003) on lysosomal membranes, the interior of these organelles is likely acidic and the charge across the membrane would be oriented positive inside. The apparent exchange stoichiometry for the zinc uptake process, as suggested by the results in Fig. 3, and by the proposed orientation of membrane potential, would be $3H^+/1Zn^{2+}$ and could be powered by both ATP and the membrane potential. Alternatively, it is possible that the asymmetric cation exchanger is not directly activated by ATP, but is instead only indirectly linked to the ATP-dependent, V-ATPase through the membrane potential it generates.

Generation of an inside positive membrane potential by the V-ATPase serves as a driving force for electrogenic anion exchange whereby cytosolic divalent or trivalent anions exchange with intralysosomal monovalent anions. Results in Figs 5, 6 and 9 suggest that two cytosolic anions (either $2SO_4^{2-}$ or $2Cl^-$) are able to exchange for single intralysosomal anions ($1Cl^-$ or $1OH^-$). In contrast, only a single divalent organic cation (1oxalate^{2-}) exchanges with a single intralysosomal monovalent anion ($1Cl^-$ or $1OH^-$). These stoichiometry differences between inorganic and organic anions may be a function of their relative hydrated radii and the fit they have at the exchanger cytoplasmic binding site. The hydrated radii of Cl^-, SO_4^{2-} and oxalate^{2-} are 0.30, 0.40 and 0.45 nm, respectively (Kielland, 1937), suggesting that the exchange stoichiometry of the transporter may convert from 2:1 to 1:1 for cytoplasmic ions possessing a hydrated radius in excess of 0.40 nm.

Once inside the lysosomal acidic interior both metals and polyvalent anions are able to increase in concentration to values in excess of those in the cytosol. Because the intralysosomal binding sites for both the $3H^+/1Zn^{2+}$ and $2SO_4^{2-}/1OH^-$ exchangers prefer monovalent ions, the transfer of either divalent metals or polyvalent anions back to the cytoplasm would be limited and would effectively trap the metals inside lysosomes in a soluble state at acidic pH. Reduction in V-ATPase activity with coincident elevation of lysosomal pH would likely precipitate metal–anion complexes and lead to the formation of a solid concretion that would detoxify the metal by lowering its availability for transfer from the lysosome. The nature of the control process(es) for variation in V-ATPase activity with resultant changes in intralysosomal pH and concretion formation is not known, but would be a fascinating extention of the present study.

The findings of this study are summarized in Fig. 10, which suggests how the hepatopancreatic lysosomal V-ATPase may power two electrogenic ion exchangers through the use of ATP and the induced membrane potential and bring about the accumulation of metals and polyvalent anions within these organelles. As shown in the figure, the asymmetric exchange stoichiometries for metal and SO_4^{2-} uptake lead to the accumulation of both ions within the organelle. It is likely that PO_4^{3-} may also enter lysosomes by the same exchanger and be available for concretion formation under the appropriate conditions (Fig. 1). Previously published experiments have shown that zinc, copper and cadmium are competitive inhibitors for transport into hepatopancreatic lysosomal vesicles (Mandal et al., 2006) and it is likely that other divalent metals and calcium are transported out of the cytosol by this mechanism. The model further suggests that high lysosomal concentrations of metals and polyvalent anions may condense into a concretion with a change in pH brought about by alterations in V-ATPase activity.

Several unanswered questions remain about the nature of concretion formation and heavy metal detoxification by the processes shown in Fig. 10. The three most significant are: (1) what is the molecular nature of the ATP-dependent, electrogenic, metal transporter that exchanges with intralysosomal protons? Is it a lysosomal isoform of the ER thapsigargin- and vanadate-sensitive calcium ATPase (e.g. SERCA) (Mandal et al., 2005)? Or is it a member of the metal transporting proteins identified for vertebrate cells such as the Znt2 or ATP7A or ATP7B (McMahon and Cousins, 1998; Liuzzi and Cousins, 2004; Vulpe and Packman, 1995; Suzuki and Gitlin, 1999)? (2) What is the physiological nature of the regulatory process(es) that control(s) lysosomal pH and

therefore regulate(s) whether enclosed metals are soluble or insoluble? (3) Are there multiple populations of lysosomes within the hepatopancreas that individually regulate the sequestration of different cations that are needed for different physiological requirements, as electron microscopic studies coupled with microprobe analysis of lysosomal contents have previously suggested (Al-Mohanna and Nott, 1985; Al-Mohanna and Nott, 1989; Hopkin, 1989; Nott, 1991)? Future experiments will be directed at elucidating some of the answers to these critical aspects of heavy metal detoxification.

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